Hormones In Human Plasma

NATURE AND TRANSPORT

Edited by

Harry N Antoniades, Ph D

Protein Foundation Laboratories Jamaica Plain
and Department of Gynecology Harvard Medical School
Boston Massachusetts

Thirty three Contributors

Foreword by

GEORGE W THORN
M D LL D S D (Hon)



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Contributors

HARRY N ANTONIADES Protein Foundation Laboratories Jamaica Plain Massachusetts and Department of Gynecology Harvard Medical School Boston Massachusetts

PAUL M BEIGELMAN Department of Medicine University of Southern California Medical School Los Angeles California

SOLOMON A BERSON Radioisotope Service Veterans Administration Hospital Bronx New York

CLIVE B CAMERON John Collins Warren Laboratories of the Collins P Huntington Memorial Hospital of Harvard University at the Massachusetts General Hospital Boston Massachusetts

JOHN F CRIGLER Jr Department of Pediatrics Harvard Medical School and the Childrens Hospital Medical Center Boston Massachusetts

YVES M DAGENAIS Baker Clinic Research Laboratory Harvard Medical School Boston Massachusetts

WILLIAM H DAUGHADAY Metabolism Division Department of Medicine Washington University School of Medicine St Louis Missouri

KRISTEN EIK NES Department of Biological Chemistry University of Utah College of Medicine Salt Lake City Utah

LEWIS L ENGEL Department of Biological Chemistry Harvard Medical School Boston Massachusetts

NORBERT FREINKEL Thorndike Memorial Laboratory Boston City Hospital and the Department of Medicine Harvard Medical School Boston Massachusetts and the Howard Hughes Medical Institute Miami Florida

ROY O GREEP Biological Research Laboratory Harvard School of Dental Medicine Boston Massachusetts

J. J. GROEN, Department of Medicine A, Hebrew University—Hadassah Medical School, Jerusalem, Israel

SIDNEY H. INGBAR, Thorndike Memorial Laboratory, Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston, Massachusetts, and the Howard Hughes Medical Institute, Miami, Florida

ERNST KNOBIL, Department of Physiology, Harvard Medical School, Boston, Massachusetts

MAURICE E. KRAHL, Department of Physiology, University of Chicago, Chicago, Illinois

HENRY D. LAUSON, Department of Physiology, Albert Einstein College of Medicine, Yeshiva University, New York, New York

MAYNARD H. MAKMAN, Department of Pharmacology, School of Medicine, Western Reserve University, Cleveland, Ohio

RICHARD S. MAKMAN, Department of Pharmacology, School of Medicine, Western Reserve University, Cleveland, Ohio

DONALD B. MARTIN, Baker Clinic Research Laboratory, Harvard Medical School, Boston, Massachusetts

JANET W. McARTHUR, Department of Medicine (assigned to Gynecology), Harvard Medical School, Boston, Massachusetts

CLAUDE J. MIGEON, Department of Pediatrics, Johns Hopkins University School of Medicine, Baltimore, Maryland

PAUL L. MUNSON, Biological Research Laboratories, Harvard School of Dental Medicine, and Department of Pharmacology, Harvard Medical School, Boston, Massachusetts

DON H. NELSON, Department of Medicine, Harvard Medical School, Boston, Massachusetts, and Peter Bent Brigham Hospital, Boston, Massachusetts *

J. L. ONCLEY, Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts

WILLIAM H. PEARLMAN, Department of Surgery, Harvard Medical School, and Peter Bent Brigham Hospital, Boston, Massachusetts

* Present address: School of Medicine, University of Southern California, Los Angeles, California

ROBERT B PENNELL Protein Foundation Laboratories Jamaica Plain Massachusetts and Department of Microbiology Harvard School of Public Health Boston Massachusetts

ALBERT E RENOLD Baker Clinic Research Laboratory Harvard Medical School Boston Massachusetts

AVERY A SANDBERG Roswell Park Memorial Institute Buffalo New York

W ROY SLAUNWHITE Jr Roswell Park Memorial Institute Buffalo New York

EARL W SUTHERLAND Department of Pharmacology School of Medicine Western Reserve University Cleveland Ohio

MARTHE VOGT Pharmacology Department University of Edinburgh Edinburgh Scotland

A F WILLEBRANDS Second Medical Service Wilhelmina Gasthuis Amsterdam The Netherlands

ROSALYN S YALOW Radioisotope Service Veterans Administration Hospital Bronx New York

J J GROEN Department of Medicine A Hebrew University—Hadassah Medical School Jerusalem Israel

SIDNEY H INGBAR Thorndike Memorial Laboratory Boston City Hospital and the Department of Medicine Harvard Medical School Boston Massachusetts and the Howard Hughes Medical Institute Miami Florida

ERNST KNOBIL Department of Physiology Harvard Medical School Boston Massachusetts

MAURICE E KRAHL Department of Physiology University of Chicago Chicago Illinois

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J L ONCLEY Department of Biological Chemistry Harvard Medical School Boston Massachusetts

WILLIAM H PEARLMAN Department of Surgery Harvard Medical School and Peter Bent Brigham Hospital Boston Massachusetts

* Present address School of Medicine University of Southern California Los Angeles California

Foreword

Delineation of the role of hormones in the maintenance of man's physical and psychological integrity as well as his capacity to adapt and reproduce has stimulated the co-ordinated efforts of chemist physiologist immunologist and clinical investigator The present volume *Hormones in Human Plasma* brings together a comprehensive and authoritative account of a seriously neglected area in human endocrinology — namely the nature of the transported hormone and its involved carrier system

Historically astute clinicians pathologists and physiologists such as Addison and Gull Graves and Basedow Berthold and Brown Séquard Von Mering and Minkowski Able and Cushing set the stage for biochemical advance in our understanding of the nature and action of hormones As might have been anticipated the most rapid strides in human studies occurred in those areas in which progress in methodology such as steroid analyses paralleled the ready availability of biological material i e the steroid content of urine Inferences regarding quantitative secretory activity of the endocrine glands in man were necessarily tentative in view of the inability to assess accurately changes in intermediary metabolism of the hormone prior to its excretion and of course the difficulties inherent in attempting to analyze blood for its microgram quantities of secretory products The major breakthrough which we are now experiencing in this area of medicine had as its cornerstone the development by Professor Edwin J Cohn and his associates of physicochemical methods for separating plasma into its various components by a technique designed to minimize alteration in chemical structure or biological activity of the contained protein constituents However even this important advance could not be fully explored until the investigator had also at his command microchemical chromatographic and immunological techniques capable of identifying extremely small quantities of hormonal substances improved microbiological assays and isotopic techniques available for identifying hormones their degradation products and the losses inherent in the analytical procedures

It is scarcely necessary to emphasize the importance of understand

where methodology is so vitally important different techniques have been presented by their own proponents The background of physical chemistry has been provided by Professor J L Oncley Professor of Biological Chemistry at Harvard Dr Robert B Pennell Director of Protein Foundation Laboratories Jamaica Plain and Dr Harry N Antoniades at present a member of both Protein Foundation and the Department of Gynecology at the Harvard Medical School

During recent years although there have been a number of conferences sponsored for the purpose of exchange of information regarding advances in endocrinology there has not been an opportunity because of limited time to discuss extensively the many aspects concerned with the nature secretion and transport of hormones in human plasma It is natural that a volume such as this should have its roots in the laboratories originally occupied by Professor Cohn It is tribute to his vision as well as his chemical skill that so many of the distinguished contributors to this volume have been in part collaborators of Professor Cohn and his associates Dr Antoniades is to be congratulated on envisioning and activating the publication of this monograph It is certain to fill a need felt by many for an authoritative statement by experts on the problems peculiar to human function For those concerned with technical advance in one small area of the field it will provide a panorama of progress and the possibility of stimulating interaction among scientists A volume of this type may be expected to stimulate research and integration at all levels including on the one hand the molecule its configuration and physical chemistry and on the other the patient exhibiting a serious physiological disability The success of the present volume will be measured by the inability ever again to contain within one single volume all information pertinent to this important subject!

George W Thorn M D LL D S D (Hon)

ing the means by which hormones are transported in the blood and the cyclical variations in plasma level which may occur throughout the 24 hours if one is to understand the role of hormones in regulating metabolic reactions The knowledge that elderly patients may exhibit a high normal blood level of thyroid hormone in the presence of subnormal secretory activity of the thyroid gland immediately suggests that the peripheral tissues of the septuagenarian may not be able to utilize thyroid hormone at a rate comparable to that in his youth By what means do estrogens increase the capacity of the plasma to trans port thyroid hormone? Is a lack of diurnal variation in the plasma level of adrenal steroids capable of inducing Cushing s disease despite a relatively normal total 24 hour secretion of 17 hydroxycorticoster oids From considerations such as these the importance of being able to determine the plasma level of hormones is evident and knowledge regarding the form in which hormones are transported in the blood is essential There is every reason to believe that the plasma concen tration of hormone is critical in regulating the rate of glandular secretion of hormone as well as its peripheral or tissue effect

The desirability of summarizing our knowledge of hormones in human plasma may be illustrated by the marked differences which are known to exist in the physiological activity of growth hormone derived from species other than man and primates In addition in man it is interesting to speculate on the potential difference between the form in which insulin exists in the peripheral blood of normal subjects after its secretion by the pancreas and passage through the liver as compared to insulin injected subcutaneously in a diabetic patient which reaches the peripheral tissues before transportation through the portal system In the case of adrenal steroids one observes the reverse normally the adrenal secretes its hormone directly into the systemic circulation whereas hydrocortisone taken by mouth must first traverse the gastrointestinal tract and portal circulation prior to reaching peripheral tissues

In organizing this volume on *Hormones in Human Plasma* Dr Harry N Antoniades has profited from his long association with important members and associates of the late Professor Edwin J Cohn at the Harvard Medical School Strength in this undertaking has been attained by selecting outstanding investigators of international repu tation who are at present contributing in an important way to prog ress in their respective fields Breadth has been attained by including investigators from countries other than the United States — and

Preface

The basic significance of experimental inquiry into blood and blood components has been recognized for centuries. As early as 1771, in his treatise on *An Experimental Inquiry into the Properties of the Blood*, William Hewson wrote:

> An inquiry into the Properties of the Blood, it is presumed, will be thought in a particular manner interesting, since there is no part of the human body upon which more physiological reasoning is founded, nor any from which more inferences are drawn for the cure of diseases. And as the inquiry is made by experiments upon the blood as near as possible to the state in which it circulates in the vessels, it is hoped that the conclusions made from them will stand the test of a candid examination, and lead to further observations and improvements.

Such an inquiry upon the blood under conditions as near as possible to the state in which it circulates was made feasible only recently, following the development of adequate tools. The ingenious work of Edwin J. Cohn (1892 – 1953) and his associates on blood and blood components and their fractionation provided invaluable new techniques and information on the composition, the preparation, the nature, and the preservation of many blood plasma components. It also provided a philosophy for the approach to problems concerned with the study of the state of plasma components under conditions close to their natural state.

These new techniques, in turn, along with the remarkably sensitive methods of hormone assay developed by biologists, provided the necessary instruments for experimental search into the state of hormones in blood plasma. Although the concept of hormones as chemical messengers that transport stimuli from tissue to tissue via the blood stream was introduced by Bayliss and Starling almost half a century ago, hormones had rarely been investigated in the state in which they circulate in the blood. In recent years, however, the concentrated effort of investigators has produced remarkable progress. Many of the hormones in human plasma have been identified. Non-protein hormones such as steroid hormones, thyroxine, epinephrine and norepinephrine appear to be transported in the blood by plasma proteins. Hormones

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of protein nature may also be in close association with other proteins in blood The physiological significance of this function is the object of current investigations Methods have been developed for the preparation of hormone concentrates from human plasma for potential clinical use

More sensitive techniques suitable for the identification and estimation of hormones in blood plasma or serum have been proposed and current efforts for the development of immunological techniques for hormone assay are most promising

Questions as to the nature of hormones and their transport mechanism in plasma both in normal and abnormal states are being eagerly explored The attempt to answer these questions has brought scientists from various fields together in a joint effort to provide an answer working under most delicate conditions in a most complex system the blood plasma

The present volume reflects this collaborative effort It hopes to provide along with authoritative scientific information the stimulus for an ever more vigorous effort in the numerous areas of this field

I am most grateful for the invaluable advice and encouragement so generously given by Dr George W Thorn in the preparation of this volume

I also wish to thank Dr Don H Nelson and Dr Albert E Renold for their kind co-operation and assistance at all times throughout this work

The excellent collaboration and understanding of the staff of the Medical Book Department of Little Brown and Company the manager Mr Fred Belliveau Mrs Mary Rackliffe and Mr Howland P Hall is greatly appreciated My thanks also to Miss Marie Gleeson for her typing assistance To my wife whose help was extended throughout this work my deepest gratitude

HARRY N ANTONIADES

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Methods of Blood Collection and Plasma Fractionation

CHAPTER I

Blood Collection

Robert B Pennell

CHANGES IN SHED BLOOD
- Serum
- Plasma

TECHNICAL ASPECTS OF BLOOD COLLECTION
- Collection of blood serum
- Use of citrate solutions for collection of plasma
- Use of ACD solutions for collection of blood
- Collection of blood through cationic exchange resins

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Much of the work discussed in this volume is concerned with *in vitro* examination of blood and its components The collection of blood and its separation into cellular elements and plasma may be accomplished in numerous ways These methods of collection and separation often influence the *in vitro* findings It is intended here to describe the various procedures in sufficient detail as to make reference to the pertinence of collection and separation techniques readily understandable

It is probable that however collected progressive changes in the character of blood begin at the time it is withdrawn from the vascular system These changes can be modified in various ways by the manner of collection The type of modification deemed most compatible with the given purpose can be directed to a certain extent by the collection procedure chosen The manner in which a given collection system may be expected to influence the blood obtained will be considered Technical details of the various methods will then be presented separately

CHANGES IN SHED BLOOD

Serum

When blood is drawn without precaution change is obvious in the formation of a clot within a short period This clot at first encom

passes both the fluid and the cellular blood components It soon begins to retract however freeing much of the fluid portion of blood as serum

Formation of the blood clot involves the transformation of one of the soluble plasma proteins fibrinogen to the solid form fibrin In this transformation a portion of the fibrinogen molecule is split off by the action of the proteolytic enzyme thrombin Thrombin is not present in blood *in vivo* but is formed in shed blood by a complex and as yet incompletely understood process usually involving a change in state of several other plasma proteins from an inactive precursor to an active form which in some instances is probably enzymic Furthermore at least one plasma protein participates in the clotting process stoichiometrically and disappears from plasma in the process The cation Ca^{++} is normally a catalyst for the beginning reactions in the clotting process

Blood serum then presents a fairly profound alteration of the fluid portion of circulating blood Little is known of the possible effects of the several enzymes formed during clotting on other proteins of the blood One cannot assume however that there are none If one can demonstrate that the particular protein of interest is unchanged by the clotting process and if one has no interest in the components of the clotting system or the cellular elements collection of serum offers the advantages of simplicity and of the greater stability of serum as compared to plasma

Plasma

One may appreciably delay the clotting of blood if in its collection it is not permitted to come in contact with wettable surfaces One of the blood cell types the platelet or thrombocyte contributes to the initiation of clotting This contribution involves the rupture of the platelet usually by contact with a rough or wettable surface Using only plastic or silicon or paraffin coated equipment blood may be drawn with little or no rupture of the platelets Such blood may remain fluid for some hours permitting the cells to be removed by centrifugation and providing an essentially unaltered plasma Such a process requires the utmost care in its accomplishment and even then clotting occurs more rapidly than is usually desirable

Most systems of blood collection assure the fluid state by the sequestration of the calcium cation This is accomplished by drawing the blood into an anticoagulant fluid or by collection of the blood

through a cationic exchange resin which retains the calcium ion Blood properly collected by any of these systems will remain fluid for some weeks at temperatures of 0° to 10°C

Of the sequestering agents citrate ion oxalate ion or ethylene diaminetetraacetate (EDTA) are most generally used in blood collection Each of these agents while delaying blood clotting by withholding calcium may also interact with other components of blood White cells are known to be adversely affected by the presence of citrate (Tullis 1953a) It is believed that prothrombin interacts specifically with citrate (Quick and Stefanini 1948 Surgenor 1953) and oxalate (Surgenor 1953) Erythrocyte glycolysis is inhibited by oxalate (Denstedt 1953) There may well be many unknown interactions Thus while retaining the clotting components relatively unchanged the plasma obtained from anticoagulant blood by centrifugation or sedimentation of the cellular elements is adulterated and altered to an unknown degree

Of the three agents mentioned citrate is by far the most commonly used Solutions of trisodium citrate alone are preferred as an anticoagulant if the blood is being drawn primarily because of the plasma and its components Collection of blood in citrate alone as it is generally performed results in an alkaline plasma approximately pH 8 The plasma is slightly hypertonic resulting in some crenation of the cellular components This is in fact an aid when the cells are removed by centrifugation the somewhat more dense cells packing more readily Erythrocytes collected in citrate alone soon exhaust the available supply of nutrient after which they lyse with steadily increasing ease If one s interest is in plasma proteins the cells should be removed from any blood as promptly as possible With citrated blood this should never be later than the fifth or sixth day following collection

By far the greatest portion of blood is collected into anticoagulant solution containing a mixture of sodium citrate citric acid and dextrose These are the so called ACD (acid citrate dextrose) solutions Although many variants of ACD have been prepared two formulas are used almost exclusively in the United States Since these are both recognized by the Division of Biologics Standards of the National Institutes of Health they have come to be known as N I H Formulas A and B They will be described as such Addition of citric acid produces an anticoagulant mixture of about pH 5 Blood collected in these solutions will yield plasma of pH 7 0 to 7 2 The inclusion of dextrose

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is salutary as far as the cellular elements are concerned permitting the erythrocytes to retain their viability for as long as 21 days at 0° to 4°C The dextrose however interacts slowly with the plasma proteins producing changes of unknown import This is most dramatically evident in albumin prepared from ACD plasma that has stood in the presence of dextrose for some time before fractionation The albumin may assume a deep red color due to an addition product with a colored derivative of dextrose

Because of its somewhat greater toxicity oxalate has not been used extensively as an anticoagulant for the collection of blood any portion of which is destined for clinical use EDTA has found its greatest use in combination with dextrose for the collection of blood to be used as whole blood Careful study has failed to show any definite advantage of such an anticoagulant over ACD and use of EDTA has never become widespread

Prevention of coagulation of blood by its collection through a cationic exchange resin has a number of theoretic advantages Unadulterated plasma is obtained Retention of the cations not only should prevent coagulation but it should halt other cation dependent enzyme actions in blood The following tabulation taken from Hughes and O Connor (1951) indicates the extent to which some of the ions are removed by collection of blood through Dowex 50 resin by the method to be described later in detail (p 9) Those metals not removed are presumed not to occur in ionic form in plasma

*Percentage Removal of Some Metal Ions from Plasma upon Collection Through Dowex 50 (Na Cycle)**

K	87%	Cu	0
Mg	97	Zn	0
Ca	99	Mn	0
Sr	93	Fe	0
Ba	92		
Al	93		

* Determined spectrographically with the exception of Zn

Sodium is exchanged for these ions resulting in a plasma pH of 7 8 to 8 0 In addition to the retention of cations certain of the blood components are now known to be retained by the resin Partial retention of blood platelets occurs (Freeman 1951 Tullis 1953b) At least one plasma *protein (the insulin binding protein)* is withheld by *the* resin (Antoniades 1958) This last observation is of particular per

tinence to the subject of this volume inasmuch as lack of recognition of retention of this protein could have led to utterly erroneous con clusions concerning the state of insulin in plasma

A final system of blood collection should be considered even though it is little used for the collection of blood in volume Heparin a sulfonated polysaccharide prevents the coagulation of blood for varying periods of time Heparin appears to achieve its action through interaction with a plasma component which has been called heparin cofactor (Astrup and Darling 1943) The complex formed is be lieved to prevent the action of thrombin on fibrinogen Thus in heparinized plasma one would expect to find all of the clotting components altered with the exception of fibrinogen with possible action of the activator enzymes upon other plasma proteins

Whatever its method of preparation plasma changes slowly during storage For this reason data describing most closely the native state are obtained when plasma is examined shortly after withdrawal of the blood Upon storage at 0 to 4 C some components of plasma complement become inactive within a few days Antihemophilic factor disappears rather quickly There is other evidence that the clotting mechanism is slowly activated and it is not unusual to find insoluble fibrin appearing within a week Still more slowly than the clotting mechanism a lytic system involving the transformation of a precursor to an active proteolytic enzyme plasmin takes place in stored plasma Many of the above changes can be avoided by holding plasma in the frozen state Freezing however produces other alterations The lipo proteins of plasma are irreversibly altered when frozen

TECHNICAL ASPECTS OF BLOOD COLLECTION

It is not the purpose here to present a detailed manual for the collection of blood nor to describe the details necessary for the preparation of blood or plasma for infusion as such Rather the main systems of collection are described only in such detail as will make reference to the system in subsequent chapters significant For the details essential to clinical use of blood or plasma *per se* one should consult the Federal Register Title 42 Part 73 Secs 73 300 through 73 306 and Minimum Requirements Human Plasma Division of Biologics Standards National Institutes of Health

Three types of receptacle are in common use for the collection of blood glass bottles glass bottles coated with silicon and plastic bags

Either type of bottle may be used with an air vent for the collection of blood by gravity or the stoppered bottle may be evacuated and the blood may be drawn into the bottle by vacuum. In any case [illegible] an anticoagulant [illegible] care must be taken to assure thorough mixing of the blood with the anticoagulant during the collection period. This [illegible] the very least, occasional [illegible] of the collection receptacle [illegible] of the receptacle. Although when great care is exercised, collection of blood by vacuum may be accomplished without rupture of any of the erythrocytes, less hemolysis is usually evident in the plasma or serum when collection is made by gravity.

For the most careful collection it is usually considered advisable to cool the blood as it is drawn. This may be done by keeping the receiving receptacle in an ice bath or other refrigerating device. It may also be accomplished by placing a coil of the tubing through which the blood is being drawn in an ice bath. Enzymatic and other interactions of the cellular elements and plasma proteins are slowed by the reduction in temperature and the changes in the shed blood are minimized.

Collection of Blood Serum

The most usual procedure for the preparation of serum is to let the blood clot as promptly as possible. To achieve this, cooling during collection is avoided and the drawn blood is allowed to stand several hours at room temperature for the formation of a firm clot. The clotted blood is then placed at refrigerator temperature for 16 to 24 hours to permit adequate syneresis. The extruded serum may be withdrawn by suction through a tube or it may be decanted carefully. To obtain maximum yield, but at the cost of some hemolysis, syneresis may be furthered by centrifugation at 2000 r.p.m. in a bucket or angle type centrifuge for an hour at 4°C.

Use of Citrate Solutions for Collection of Plasma

Blood collected primarily for the preparation of plasma is usually drawn into an [illegible] anticoagulant solution of the following composition:

Trisodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) — 4.00 gm
Water for injection (U.S.P.) to make 100 ml

A volume [illegible] ml of this solution for [illegible]

drawn is added to the receiving receptacle *before sterilization* Assuming plasma to represent half the volume of whole blood adherence to the above *proportions results in a plasma concentration of* essentially 0 46 per cent or 24 8 millimolar citrate Variations of this *technique yielding plasma concentrations as low as 15 millimolar* citrate are current in parts of Europe

Separation of cells from plasma may be achieved after centrifugation or after permitting the cells to sediment spontaneously After *collection and during storage* and separation the blood should be held at 4° to 10 C

Use of ACD Solutions for Collection of Blood

By far the largest portion of blood collections is made into acid citrate dextrose anticoagulant solutions Such blood may then be used as whole blood or may be separated into cells and plasma

Although many variants of ACD solutions have been proposed only the two commonest formulas need to be considered here

Ingredients	*N I H Solution A*	*N I H Solution B*
Trisodium citrate ($Na_3C_6H_5O_7$ $2H_2O$)	22 0 gm	13 2 gm
Citric acid ($C_6H_8O_7$ H_2O)	8 0 gm	4 8 gm
Dextrose ($C_6H_{12}O_6$ H_2O)	24 5 gm	14 7 gm
Water for injection (U S P) to make	1000 ml	1000 ml

For each 100 ml of blood to be drawn 15 ml of Solution A or 25 ml of Solution B are added to the receiving unit before sterilization If the volumes are observed with some accuracy and again assuming plasma to comprise one half the total volume of blood Solution A gives plasma concentrations of 29 4 millimolar citrate and 230 millimolar dextrose whereas Solution B yields 26 8 millimolar citrate and 148 millimolar dextrose The plasma obtained when Solution B is the anticoagulant is of course appreciably diluted

Collection of Blood Through Cationic Exchange Resins

Either sulfonated or carboxylated cationic resins may be used for the sequestration of the cations of blood Specifications of resins suitable for this purpose (Gibson 1951) include the following

Particles should be spheroidal Particle size should be 20 to 40 mesh Cross linking should be 8 to 12 per cent for either sulfonated or carboxylic resin There should be the minimum possible extractable

solubles either before or after autoclaving Exchange capacity should be such that when 500 cc of the following test solution is passed through 50 cc of wet resin (meshed with 0 15 M NaCl) in a column 1 inch in diameter the total effluent Ca^{++} and Mg^{++} should not exceed 5 per cent of the concentration of these ions in the influent test solution

Test Solution
To Be Adjusted to pH 7 4 with HCl

	mEq L
Ca^{++}	5 00
Mg^{++}	2 00
K^{+}	5 00
Na^{+}	140 00
Cl^{-}	100 00
Acetate^{-}	52 00

These specifications are met by Dowex 50* and IRC 50† resins of proper mesh the two most commonly employed resins for blood collection

Fifty cubic centimeters of wet resin is adequate for the collection of 500 cc of blood at a rate of 1 cc of blood per cubic centimeter of resin per minute provided the height of the resin column is not less than 13 cm (Buckley *et al* 1950) The proper rate of blood flow is best achieved by collection by gravity The resin on the sodium cycle should be buffered at pH 6 5 with sodium phosphate monobasic (NaH_2PO_4) and meshed with 0 15 M NaCl

When resin collection is coupled with plastic or properly surfaced equipment (Walter 1950 Walter *et al* 1951) and is accompanied by simultaneous separation of cells and plasma (Tullis *et al* 1956) under refrigeration one probably obtains plasma most nearly unchanged from its natural state

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*Plasma Proteins and Plasma Fractionation**

J L Oncley

CONCENTRATION AND PROPERTIES OF SOME PLASMA PROTEINS

CRITERIA OF PURITY

GENERAL PRINCIPLES OF PROTEIN FRACTIONATION

SALTING-OUT FRACTIONATION METHODS

COLD-ETHANOL FRACTIONATION METHODS

PROTEIN FRACTIONATION WITH ZINC

COUNTERCURRENT DISTRIBUTION AND CHROMATOGRAPHIC METHODS

- Countercurrent distribution
- Liquid liquid (partition) chromatography
- Solid liquid chromatography

ELECTROPHORETIC FRACTIONATION METHODS

ULTRACENTRIFUGAL FRACTIONATION METHODS

BIBLIOGRAPHY

The isolation and purification of proteins is a highly complex procedure much more an art than a science Proteins occur in nature as complex mixtures and are often found in the presence of highly charged polysaccharides and/or other macromolecules These naturally occurring colloidal mixtures are often enclosed by membranes of varying stability The extraction of a particular protein from such a mixture is always difficult and is often impossible without the use of procedures which may cause permanent changes in the configuration or even in the covalent linkages of the resulting protein preparation

In the case of the plasma proteins there do not appear to be high concentrations of charged polysaccharides or nonprotein macromolecules Also we do not have membranes to complicate the picture and all of the protein molecules are already in a state of solution Even in this simpler form however the problem of isolation and purification

* Part of this work was supported by grants from the National Heart Institute (H 2127) and the Life Insurance Medical Research Fund (No G 58-7)

is a formidable one and much labor has been expended in the for mulation of systems for the complete fractionation of the plasma from various sources

CONCENTRATION AND PROPERTIES OF SOME PLASMA PROTEINS

Plasma is composed of many protein components the majority being present only in trace amounts Numerous reviews of the blood plasma proteins exist (Cohn 1941 1947 Pedersen 1945 Edsall 1947 Grabar 1947) the review of Hughes (1953) is one of the most recent The short summary presented here may be helpful in our under standing of the problems involved in the design of plasma protein fractionation methods

TABLE I Some Protein Components of Human Plasma*

Component	Conc tr tion gm/l	% I Plasm protein	Aup i m te pI	S dimen t tion sta t† $s_{20\,w} \times 10^{13}$	El ctr phor tic mpn e t‡	M l ul r w ight
Alb min	33 6	51 0	4 9	4 6	A	69 000
γ Globulins	6 7	10 0	6 3 7 3	7 0	γ	156 000
Fibrinogen	2 7	4 5	5 3	7 9	β_2	340 000
α Lipoprot in (1 09 1 14)	2 6	4 0	5 2	4 6	α_1	1 6 4 0 x 10^5
β Lipop otein (P 1 03$_2$ ± 0 07)	2 5	3 8	5 4	6	β_1	2 x 10^6
β_1 Metal c mbi ing protein	0	3 0	5 8	5 0	β_1	90 000
β_2-Globulins	2 0	3 0	6 3	7 0	β_2	(150 000)
β_1 Lipid poor euglobulin (s 7)	1 3	2 0	5 5	7 0	β_1	(150 000)
β Lipoprotein (ρ 1 01)	1 0	1 5	5 4	1 8	β_1	3 x 10^6
α_2-Glyc protein	0 8	1 2	4 9	9 0	α_2	(300 000)
γ Globulins (s 18)	0 3	0 5	5 6	18 0	γ	ca 1 x 10^6
β_1 Lipid poor euglobulin (s 18)	0 3	0 5	5 4	18 0	β_1	ca 1 x 10^6
α_2-Mucoproteins	0 3	0 5	4 9	9 0	α_2	(300 000)
α_1 Acid glycoprotein	0 3	0 5	3 0	2 9	α_1	45 000
Caeru opla mi	0 3	0 5	4 4	7 2	α_2	150 000
C mpl ment compon nts (C 1 C 2 C 3 C 4)	0 3	0 5	—	—	—	—
Cold ins luble globulin	0 10	0 15	5 3	13	β_2	(00 000)
Pr thrombin	0 09	0 13	5 0	4 8	α_1	63 000
Bilirubin-contai i g gl bulin	0 03	0 05	4 7	—	—	—
Anti A anti B is agglutinins	0 02	0 03	—	—	—	—
Serum esterase	0 006	0 01	4 4	(12 0)	(α_2)	(300 000)
Diphtheria a titoxin	0 0006	0 001	(7 0)	(7 0)	γ	(150 000)

* Revised f om the ta les tim te publi hed by Cohn (1947) Coh et al (1950) and by Oncley et al (1947)
† Meas red in 0 15 M N Cl lution
‡ M red i pH 8.6 Γ/2 0 1 diethyl barb iturate buff r

Table I modified from earlier tables of Cohn (Cohn 1947 Cohn *et al* 1950) and Oncley (Oncley *et al* 1947) lists some of the better

known protein components of human plasma Ten proteins make up about 84 per cent of the plasma proteins and the next ten or twelve most abundant proteins bring this total up to perhaps 88 per cent Many components are known to be present in amounts less than 0 1 per cent of the total plasma protein concentration (less than 0 7 gm per liter) * Indeed most of the components discussed in this volume are present to less than this amount There would thus appear to be many hundreds of protein components present in plasma and these trace components are often of great importance to the normal function of the body

When dealing with such trace components it is especially difficult to determine the state of purity of isolated fractions high in biological activity Often it is difficult or impossible to fractionate the large volumes of plasma necessary to obtain reasonable amounts of protein for chemical and physical-chemical study It is also difficult to establish the fact that a homogeneous appearing material contains even a major amount of the biologically active component If this is not the case it is easy to characterize a protein which has no relationship at all to the biologically active material

CRITERIA OF PURITY

We will not be able to critically discuss the criteria of purity of protein preparations The classic work of Sumner and of Northrop and their colleagues in proving the protein nature of certain enzymes and demonstrating the homogeneity of preparations of several enzymes should be studied by all workers interested in the purification of trace protein components This work is reviewed by Northrop *et al* (1948) More recent reviews by Li (1951) and by Edsall (1953) should also be consulted

Methods available for the demonstration of protein homogeneity include electrophoresis ultracentrifugation and diffusion In using these methods it is important to demonstrate that the biologically active component moves with the same rate as that found for the principal protein component The solubility method where it can be demonstrated that the solubility (as measured both by activity and

* The estimates of the concentration of prothrombin serum esterase and diphtheria antitoxin are made with the assumption that the activity of these components is the same in human plasma as were the materials purified from animal plasmas and that the highest concentrations which have been reported represent the completely purified component

by protein concentration) is independent of the concentration of the saturating body is especially powerful and can be thought of as replacing the characterization of simpler organic molecules by means of the freezing point or boiling point of the pure compounds Here it is important to study the solubility at several different ionic strengths and pH values to eliminate the chance of an accidental identity of solubility This situation is a likely one when the solubility study is made at conditions near those used for the isolation of the prepara tion Immunological assay is also a very useful method for deter mination of purity and this method is often combined with diffusion or electrophoresis studies to improve their resolution The smaller proteins can occasionally be studied by the method of countercurrent distribution and elution from various types of supporting media has proved of considerable usefulness Few if any proteins have met all of these tests for homogeneity But even more troublesome is the fact that several proteins have been claimed to be pure whereas subse quent studies have indicated that the biologically active material is probably only a trace impurity of an inactive but reasonable homo geneous protein carrier !

GENERAL PRINCIPLES OF PROTEIN FRACTIONATION

The careful control of various physical chemical conditions during protein separation and purification is of great importance The gen eral principles which should be observed have been discussed often recent reviews of Fevold (1951) and Taylor (1953) may be useful and a careful discussion of this problem will be found in Cohn and Edsall (1943) Factors to be considered include

(1) *Temperature* Temperatures should be maintained at as low a level as possible Room temperature is too high for most stages in protein purification The use of thermal denaturation of less stable components in protein purification must be employed with discretion and evidence that the elevated temperatures have not caused changes in even the stable protein must be presented Low temperatures also retard the growth of microorganisms and the action of destructive enzymes which may be present

(2) *pH* Since most proteins are stable only in a rather narrow pH range *it is important to avoid pH conditions much removed from the* natural environment of the native protein

(3) *Protein concentration* Highly diluted protein solutions are

often unstable This is especially true if large amounts of interface are present (such as foam or large filtration interfaces) In general it is most useful to work with solutions containing from 1 to 50 gm per liter

(4) *Organic solvents* Organic solvents with dielectric constant lower than water must be used with caution and at as low a temperature as possible It is also important to carefully stir the solution during the addition of such materials to avoid local excess concentrations and to dissipate the heat of mixing

(5) *Stabilizing agents* Protein solutions are often stabilized by the addition of small molecules Dilute solutions of neutral salts often show this stabilizing effect and certain substances can often be found to have a maximum stabilizing effect

(6) *Drying* The process of drying is best carried out by the sublimation of the solvent from the frozen solution Not all proteins can be treated in this manner without danger of denaturation This seems especially true of certain of the lipoproteins Negative pressure dialysis is a useful technique for the concentration of proteins which cannot be safely dried Well dried proteins (less than 0 5 per cent moisture) are usually stable for long periods of time Proteins containing several per cent of moisture are often quite unstable upon storage

(7) *Trace metals* Traces of heavy metals often cause the denaturation of proteins It is important to see that such contaminants are not introduced through the use of impure reagents or by metal equipment coming in contact with the protein solutions When heavy metals are used for protein fractionation they usually should be removed by ion exchange columns or by dialysis against solutions of effective chelating agents before drying the protein solutions

Even when the mildest methods are used for the isolation or concentration of a particular trace plasma protein it is difficult to prove that its molecular state is identical with its state in nature to borrow a phrase emphasized by E J Cohn (1947) For example there is considerable evidence much of it presented in other chapters of this book for the existence of complex protein forms of insulin most or all of the peptide hormones vitamin B_{12} etc The isolation of the simplest forms of these vitamins and hormones has been of great importance both to their use in therapy and to the better understanding of their mode of action However the isolation of the more complex forms of these biologically active materials is a necessary step in our understanding of their action and here it is of great importance

to use only the most carefully controlled conditions for the isolation of these forms of the protein There is often a mistaken view that methods involving simple procedures are more gentle but this is certainly not necessarily true For example a complex procedure of protein fractionation which maintains low temperatures and narrow ranges of acidity or alkalinity is probably more likely to isolate a native complex than a chromatographic or electrophoretic method involving higher temperatures or more extreme pH conditions The proof that any given complex is similar or identical with that found in the plasma or other biological tissue is very difficult to establish and thus the use of only procedures which are known to be least likely to produce structural alteration has a great advantage

SALTING OUT FRACTIONATION METHODS

The oldest methods for the isolation and purification of the plasma proteins depended upon the salting out methods using high concentrations of ammonium or sodium sulfate in order to precipitate the various proteins present The most recent reviews dealing with this problem are those of Cohn *et al* (1940b) Cohn (1941) Edsall (1947) Grabar (1947) Fevold (1951) and Taylor (1953) The most recent comprehensive study of this problem is the thesis of Pedersen (1945) Only a few highly purified proteins have been isolated from serum or plasma by the salting-out methods Pedersen (1945) states that it is not an easy matter to prepare pure serum proteins by fractionation with ammonium sulphate

The salting out methods have defined the classic albumin euglobulin and pseudoglobulin fractions of serum The following definitions are taken from Fevold (1951)

Albumins are heat coagulable proteins which are soluble in water dilute salt solutions and dilute acids and bases They are also characterized by being more soluble in concentrated salt solutions than other proteins concentrations of 2 molar (0 5 saturation) ammonium sulfate or above being necessary for their precipitation

Globulins are insoluble in pure water at their isoelectric points but are brought into solution by low concentrations of salts and by dilute acids and alkalies In general they are precipitated from salt solutions more readily than are albumins (below 2 0 molar ammonium sulfate) Globulins are sometimes divided into two groups *euglobulins* which are the globulins as defined above and *pseudoglobulins* Pseu

doglobulins are characterized by being precipitated at a concentration of 2 0 molar ammonium sulfate and by not being insoluble at the iso electric point The pseudoglobulins therefore may be termed a transition class between albumins and globulins and could as well be classified as albumins which are somewhat less soluble in concentrated salt solutions Methods are available for the preparation of purified albumin (carbohydrate free) (McMeekin 1939) carbohydrate containing albumin (probably better classified today as an α_1-glycoprotein) (McMeekin 1940) γ-pseudoglobulin (Cohn *et al* 1940b) and fetuin (Pedersen 1944)

Table II from the data of Pedersen (1945) summarizes the principal types of plasma proteins precipitated under typical concentrations of ammonium sulfate Table III lists some of the better known salt fractions with approximate electrophoretic characteristics *

COLD ETHANOL FRACTIONATION METHODS

The most extensive scheme for the fractionation of human plasma is the cold-ethanol procedure developed during World War II (Cohn *et al* 1940a 1946) Certain advantages and dangers in the use of these methods are carefully outlined by Edsall (1947) By the use of Method 6 for the fractionation of plasma (Cohn *et al* 1946) and the various subfractionation methods developed for Fractions I (Morrison *et al* 1948) II + III (Oncley *et al* 1949) IV-4 (Surgenor *et al* 1949) V (Cohn *et al* 1947 Hughes 1947) and VI (Schmid 1955) it is possible to subdivide the plasma proteins into about 20 fractions Although these 20 subfractions do not represent homogeneous protein fractions they do divide the various protein components into fractions containing from 0 6 to 13 per cent of the total plasma protein (excluding the albumin containing Fraction V 48 per cent of the plasma protein) and many of the biologically or chemically defined protein components are present in good yield in a single fraction Much of the value of these methods depends upon the vast amount of work which went into the development of these procedures (roughly estimated to be of the order of 500 000 man hours of research time) The cold ethanol method often referred to as a five variable system (the variables being pH ionic strength ethanol concentration protein concentration and temperature) has the advantage of having more

* None of these fractions appears to be completely electrophoretically homogeneous However they do seem to contain mainly the electrophoretic components listed

TABLE II Principal Types of Plasma Proteins Precipitated under Typical Concentrations of Ammonium Sulfate*

Concentration of ammonium sulfate m/l	% sat	pH	Principal types of proteins precipitated	% of Total plasma protein
1 10	0 33	7 0	fibrinogen, γ globulins	32
1 64	0 40	7 0	γ globulins β globulins	8
1 85	0 45	7 0	β-globulins α globulins	8
2 06	0 50	5 0	β globulins, α globulins	4
2 28	0 55	5 0	Albumin	23
2 47	0 60	5 0	Albumin	20
4 10	1 00	5 0	Albumin	5

* Adapted from Pedersen (1944)

TABLE III Principal Electrophoretic Components of Some Fractions of Plasma Obtained by Salting out Methods

Fraction	Principal electrophoretic component
P_I (Green 1938)	α
Euglobulin II (Hewitt 1934)	
P_{III} (Green 1938)	β
P_{II} (Green 1938)	γ
Euglobulin I (Hewitt, 1934)	β and γ
Carbohydrate containing albumin (Kekwick, 1938) (McMeekin, 1940)	α_1
Carbohydrate free albumin (Hewitt, 1936) (Kekwick, 1938) (McMeekin, 1939)	Alb

possible sets of conditions for satisfactory fractionation and yet is not so complex that the development of a satisfactory system is an excessively difficult task The salting-out system contains too few variables

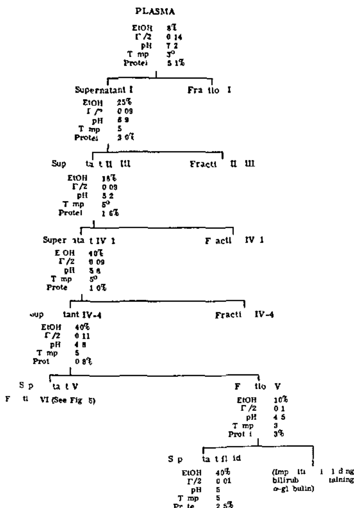


FIGURE 1 Method 6 for the fractionation of human plasma (From Cohn *et al* 1946)

when viewed in this way and the fractionation methods involving metals for example Method 12 (Surgenor *et al* 1960) are somewhat too complex The additional variables in the metal systems are necessary to further develop adequate fractionation methods for a mixture of proteins as complex as plasma but the man hours of research time which must be expended for a complete system (perhaps several mil

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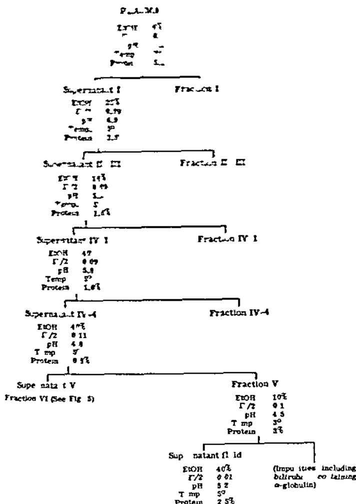


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tion) are considerably beyond the capabilities of any group at this time

Figures 1 through 5 consist of abbreviated flow diagrams for these methods (Method 6 and subfractionation of cold-ethanol fractionation) Detailed instructions for conducting these procedures are in the original references (Cohn *et al* 1946 1947 Hughes 1947 Morrison

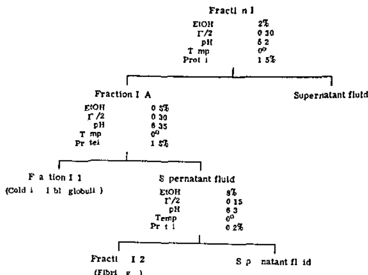


FIGURE 2 Subfractionation of Fraction I (Outlined from Morrison *et al* 1948)

et al 1948 Oncley *et al* 1949 Surgenor *et al* 1949 Schmid 1955) The yields obtained when pools of normal human plasma are used are recorded in Table IV Another method for the subfractionation of Fraction II + III has been reported (Deutsch *et al* 1946) and a useful rework of Fraction I has been published (Blomback and Blomback 1956) A chromatographic separation of Fraction VI has also been developed (Schmid *et al* 1958) Further subfractionation of certain of these fractions has been studied Rework of Fraction III 0 has been reported by Oncley *et al* (1950 1957) and by Antoniades *et al* (1957) Kline (1953) has fractionated Fraction III to obtain plasminogen Surgenor and Ellis (1954) have reworked Fraction IV 6 to obtain subfractions high in serum esterase activity and a subfractionation of Fraction IV 7 to obtain crystallized β metal combining protein has been reported by Koechlin (1952) and by Inman and Oncley (1959)

A number of earlier procedures (Methods 1-5) for the initial frac

F ctl n II III
EtOH 20%
Γ/2 0 005
pH 7 2
T mp 5
Prot i 1%

S p ta t fl id
EtOH 25%
Γ/2 0 005
pH 5 7
T mp 5
P t i 0 4%
C (A)$_2$ b ff

F ctlon III O
(β Lipoprot i)

S p t t fl id
(Pr tei poo)

F cti n II IIIW
EtOH 17%
Γ/2 0 015
pH 5.2
T mp 6°
Pr t i 1 2%

Fr ction III
EtOH 1%
Γ/2 0 09
pH 5 4
T mp 0
Prot i 2 5%

F actio III 2 3
EtOH 0%
Γ/2 0 1
pH 7 0
T mp 0
P t i 6%

Fra tio III 3
(Plasmi g)

S p tant fl id

Lypholi ti

F ti III 2
(P th mbi)

S p r atant fl id
EtOH 17%
Γ/2 0 06
pH 5 2
T mp 6°
Prot i 0 5%

Fractio III 1
(Is aggl tini)

Sup atant fl id
(Protei poo)

S pernatant fluid
EtOH 17%
Γ/2 0 05
pH 5 2
T mp 6°
Protei 0 8%

Supe tant fl id
EtOH 25%
Γ/2 0 05
pH 7 4
T mp 6°
Pr t i 0 4%

Supernata t fl id
(Prot i poo)

Fr ction II 1 2
(γ Gl bulins)

Fracti n II 3
(γ Globuli)

FIGURE 3 Method 9 for the subfractionation of Fraction II + III (Adapted from Oncley *et al* 1949)

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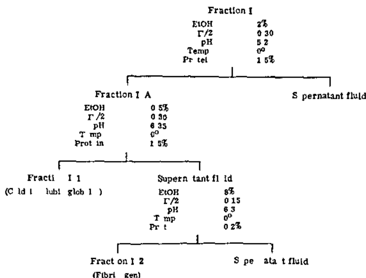


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A number of earlier procedures (Methods 1–5) for the initial frac

tionation of plasma were also reported by Cohn *et al* (1946) A recent modification of Method 5 has been of considerable value in the com mercial production of albumin γ globulin and fibrinogen and is known as Method 5H A flow diagram of this procedure is shown in

TABLE IV Estimated Distribution of Plasma Proteins into Subfractions by *Cold Ethanol Fractionation (grams per liter of original plasma)*

Fraction	A	1	α_2	β_1	β	ϕ	γ	T tal
I 1	0	0	0	0 15	0 1	0 45	0	0 7
I 2	0	0	0	0 15	0 1	1 25	0	1 4
S p natant I A	0 2	0 15	0 1	0 05	0 05	0 1	0 25	0 9
Supernatant I 2	0	0	0 05	0 05	0 05	0 2	0 05	0 4
I (Total)	0 2	0 15	0 15	0 3	0 3	2 0	0 3	3 4
II	0	0	0	0	0 1	0	5 4	5 5
III 0	0 5	0 2	0 3	5 7	1 1	0 2	0 4	8 4
III 1	0 1	0 2	0 3	0 4	1 1	0 1	0 4	2 6
III 2	0	0 1	0 3	1 0	0 1	0 1	0 1	1 7
III 3	0	0	0 2	0 2	0 1	0 3	0	0 8
II III (Total)	0 6	0 5	1 1	7 3	2 5	0 7	6 3	19 0
IV 1	0 5	3 0	1 0	0 4	0 1	0	0 1	5 1
IV 5	0 05	0 65	0 15	0 05	+	0	0	0 9
IV 6	0 1	0 15	1 1	0 05				1 4
IV 7	0 15	0	0 1	0 75	0	0	0	1 0
IV 8	0 9	0 04	0 03	0 03	0	0	0	1 0
IV 9	0 45	0 01	0 42	0 6	0	0	0	1 5
IV-4 (Total)	1 65	0 85	1 8	1 5	0	0	0	5 8
V	29 9	0 9	0 4	0 3	0	0	0	31 5
VI Ia	0	0 004	0 091	0 005	0	0	0	0 10
VI Ib	0	0 374	0 016	0	0	0	0	0 39
VI 2	0	0 0600	0 010	0	0	0	0	0 08
VI 3	0 34	0 03	0	0	0	0	0	0 37
VI (Total)	0 34	0 47	0 12	0 005	0	0	0	0 94
Pl ma (Total)	33 2	5 9	4 6	9 8	2 9	2 7	6 7	65 8

Figure 6 and is included since many of the fractions obtained through the co operation of the American Red Cross Blood Fractionation Program have been prepared by this procedure

A modification of the cold ethanol method using a quite different set of original fractionation conditions has been developed and is known as Method 10 (Cohn *et al* 1950 Lever *et al* 1951) It uses considerably lower protein concentrations and ionic strengths and attempts to take advantage of certain protein protein and protein metal interactions It also introduces the method of fractional extrac tion as contrasted to the usual method of fractional precipitation used in Method 6 Method 10 presents advantages in the fractionation of certain of the more labile plasma protein components probably because of the use of somewhat shorter equilibrium times and lower ethanol concentrations Figure 7 gives a flow diagram of a slight mod ification of this method known as Method 10B in current use at the

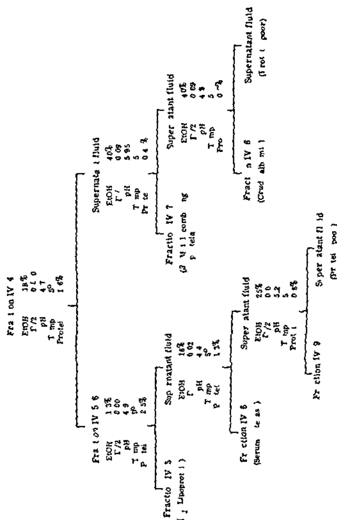


FIGURE 4 Rework of Fraction IV 4 (Adapted from Surgenor *et al* 1949)

solubility of about 18 5 volumes per 100 volumes of aqueous phase at −4°C The low dielectric constant of ethyl ether partially compensates for this low solubility however

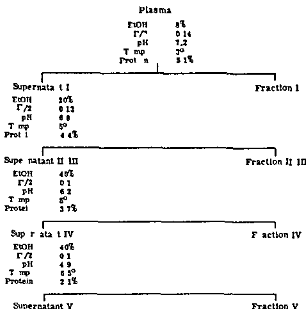


FIGURE 6 Method 5H for the fractionation of human plasma (Adapted from Gerlough 1954)

PROTEIN FRACTIONATION WITH ZINC

The plasma proteins interact reversibly with heavy metals at physiologic pH The interactions with the cations of zinc lead mercury and cadmium appear to be most useful for the fractionation of the plasma proteins (Cohn *et al* 1953) and of these zinc has been studied the most The rate of reaction is rapid and separations between soluble and insoluble zinc protein complexes can often be carried out after 20 to 30 minutes of standing at 0 C The zinc can be removed from such complexes by passage over ion-exchange resins thus providing a rapid method for the isolation of plasma protein fractions and avoiding the necessity of a drying cycle

The addition of zinc acetate causes a drop in the pH of the protein solution due to the release of H^+ upon the binding of Zn^{++} This pH change can usually be prevented by the use of mixtures of zinc acet

Protein Foundation Laboratories (Melin *et al* 1959) Method 10B differs from the published Method 10 procedures in the precipitation of a Fraction I using a greater dilution than was used in Method 6 A method for plasma fractionation involving principles similar to Method 10 has also been developed by Nitschmann (1954)

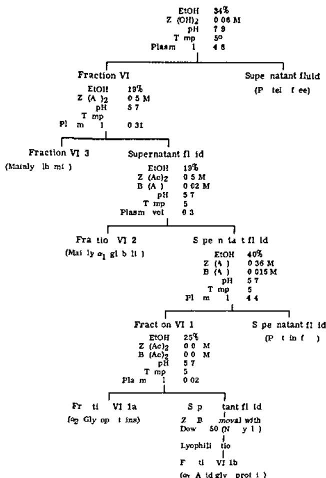


FIGURE 5 Separation of the proteins precipitated in Fraction VI (Adapted from Schmid 1954)

Modifications of the cold ethanol method involving the use of methanol (Pillemer and Hutchinson 1945) and ethyl ether (Kekwick and Mackay 1954) have been developed The use of these other organic solvents is based on the hope that they may cause less destruction of certain labile proteins Ethyl ether suffers from the disadvantage that it is not completely miscible with water with a maximum

solubility of about 18.5 volumes per 100 volumes of aqueous phase at −4°C. The low dielectric constant of ethyl ether partially compensates for this low solubility, however.

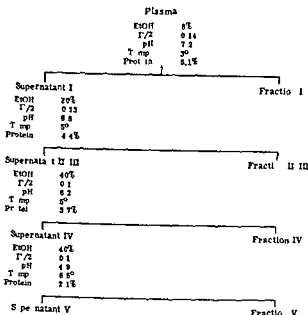


FIGURE 6. Method 5H for the fractionation of human plasma. (Adapted from Gerlough, 1954.)

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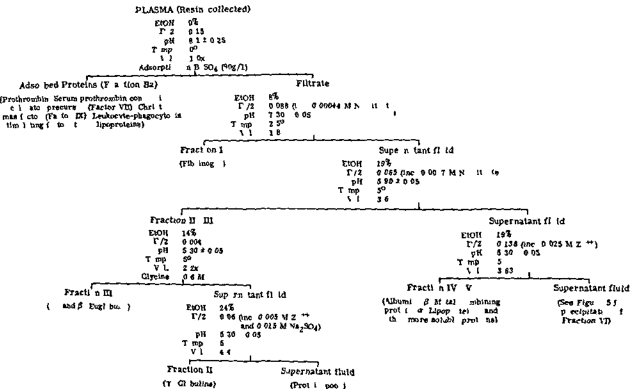


FIGURE Method of 10 R for the fractionation of human plasma (Melin *et al* 1959)

and zinc glycinate * It is usually found that there are certain zinc concentrations where the further addition of zinc causes relatively small additional protein precipitation These conditions are especially useful for the rapid precipitation of reproducible protein fractions The fractional extraction of such precipitates using a series of rea gents with increasing affinity for zinc ions affords conditions where rapid extractions of specific proteins can be carried out

Such a procedure for plasma fractionation also utilizing the more or less specific adsorption of certain proteins upon barium sulfate starch etc. has been developed (Surgenor *et al* 1960) This fractiona tion system often referred to as Method 12 appears especially useful for the preparation of certain labile plasma proteins especially those involved in the clotting of fibrinogen and allows the rapid prepara tion of a solution containing nearly all of the serum albumin together with about 15 per cent of α globulins and β-globulins which can be heated for many hours at 60°C without appreciable deteriora tion (known as SPPS – stable plasma protein solution) Procedures are available for the separation of most of the clotting components β lipoproteins and γ globulins A flow diagram for this method is shown in Figure 8

COUNTERCURRENT DISTRIBUTION AND CHROMATOGRAPHIC METHODS

The usefulness of chromatography and countercurrent distribution in the separation of components of lower molecular weight has led to the desire to apply these methods to protein mixtures Because of the large size and limited stability of the protein molecules it has been difficult except for a few of the smaller and more stable pro teins to find media with sufficient capacity to make the chromato graphic methods useful It has also been difficult to find two-phase solvent systems where both phases give finite solubility for many pro teins

Countercurrent Distribution

This method more or less equivalent to the simple extraction in the chemical laboratory using a series of separatory funnels and a two

* Zinc glycinate as the salt $Zn(NH_2CH_2COO)_2$ can easily be prepared by the addition of freshly precipitated zinc hydroxide to a hot glycine solution This salt can be crystallized from 0.5 molar solution upon cooling

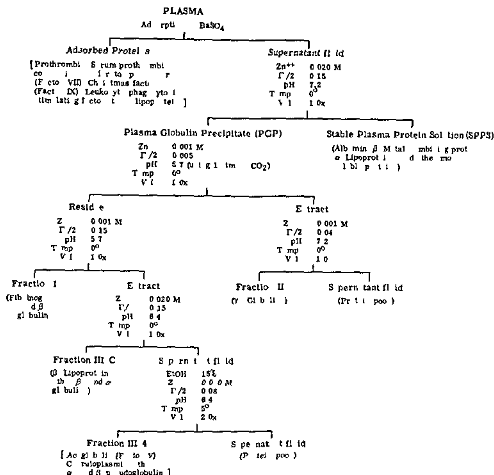


FIGURE 8 Method 12 for the fractionation of human plasma (Adapted from Surgenor *et al* 1960)

phase distribution system has been developed into a very powerful fractionation method by Craig through the introduction of mechanical devices for automatically repeating this process hundreds or thousands of times (Craig and Craig 1950) Application of this method to proteins is limited by the difficulty of obtaining satisfactory two phase systems since most immiscible phases either cause denaturation of the proteins or do not result in much distribution into one of the phases Applications of the method to protein systems have recently been reviewed by von Tavel and Signer (1956) and by Craig and King (1959) It has been applied to insulin ribonuclease lysozyme bacterial catalase and casein The only plasma protein studied so far has been serum albumin (Hausmann and Craig 1958 Craig and King 1959)

Two solvent systems have been used in these albumin studies the two phase system resulting from the use of trichloroacetic acid acetic acid 2 butanol ethanol and water gave a hyperfine (self sharpening) elution curve broadening to nearly the theoretical curve upon dilution of the protein (Hausmann and Craig 1958) whereas the two-phase system resulting from the use of sodium caprylate ammonium sulfate propanol ethanol and water gave several peaks of a more normal type (Craig and King 1959) These systems were used at about 25°C and denaturation was minimized by the introduction of the trichloroacetic acid or the sodium caprylate both stabilizing agents for serum albumin

The various chromatographic methods have been categorized in terms of the stationary and mobile phases used and in terms of the method used in contacting these phases A recent review of the fundamentals of chromatography by Cassidy (1957) sets forth the various methods and materials available for chromatographic work and discusses the theoretical basis of the methods It is convenient to divide the separation of chromatographic systems into liquid liquid chromatography usually called partition chromatography and solid liquid chromatography often subdivided into adsorption chromatography and ion-exchange chromatography It is also useful to classify the *methods used for the development of the separations as starting agent* development (sometimes called frontal analysis) where the same solution is used for both the column equilibration and development gradient development where the development agent is continuously changed and stepwise development where a series of development agents are used This is a classification recently outlined by Boman (1958) in a valuable review of protein chromatography More comprehensive reviews of protein studies have been written by Moore and Stein (1956) and by Sober and Peterson (1957)

Liquid liquid (Partition) Chromatography

The partition chromatography methods are closely related to the method of countercurrent distribution of Craig the column packing material being primarily of importance in holding one of the liquid phases In most cases the aqueous phase is immobilized by such column material as kieselguhr (Hyflo Super Cell or Celite) while the organic phase is run through the column Alternatively the organic phase is held stationary by saline treated kieselguhr for example while the aqueous phase is mobile Such hydrophobic adsorption

systems are often called reversed phase partition chromatography The application of partition chromatography to proteins is reviewed by Porter (1955a) It has been applied to insulin and to a number of enzymes The only plasma protein system extensively studied by this method appears to be rabbit γ globulin studied by Porter (1955b) and by Humphrey and Porter (1956) A two phase system using water potassium phosphate (pH 9) and ethyl and butyl cellusolves was used with the temperature held at $-3\ 1 \pm 0\ 05°C$ to minimize denatura tion The aqueous phase was held on Celite 545 Broad elution curves were obtained which appeared to arise from the presence of a con siderable number of similar components since rechromatography of narrow cuts yielded reasonably sharp elution curves of consistent R_f value

The serum proteins have also been studied by paper chromatogra phy using a phenol water mixture with and without the addition of butanol or acetic acid (Grassmann and Deffner 1953) There was con siderable evidence of denaturation using these solvents however

Solid liquid Chromatography

The original chromatographic method of Tswett involved pri marily the adsorptive distribution between a fluid mixture and the surface of a nonionic solid adsorbent In ion exchange chromatogra phy the adsorptive process is ionic and the adsorbent a polyelectro lyte Most solid adsorbents have some of both of these properties and it is difficult to classify them precisely into these two processes unless the adsorbent is some form of carbon or a neutral carbohydrate such as starch unmodified cellulose etc The modified (ionic) cellulose derivatives introduced by Peterson and Sober (1956) or even the commercial ion exchange resins having a polystyrene or other hydro carbon skeleton usually will adsorb proteins with forces of both ionic and nonionic character

Adsorbents most useful for the chromatographic fractionation of proteins usually have considerable ionic character since it is difficult to control the elution characteristics without the use of nonpolar sol vents undesirable for use in protein containing systems The most useful inorganic adsorbents have been the hydrated oxides of silicon or aluminum calcium phosphate gels barium sulfate crystals mont morillonite and kieselguhr Organic adsorbents have included various forms of carbon nonionic carbohydrate materials such as starch and

cellulose but the most useful types of materials have proved to be the synthetic ion-exchangers and a series of modified cellulose ion exchange adsorbents The synthetic materials most useful included the weakly acidic (carboxylated) Amberlite IRC 50 (and especially XE 64 a fine mesh material of the same type) the strongly acidic Dowex 50 (sulfonated) or Amberlite IR 120 the weakly basic Amberlite IR-4B Amberlite IR-45 and Dowex 3 and the strongly basic Dowex 2 and Amberlite IRA-400 Tables describing these commercially available materials will be found in a recent review by Kressman (1957) The modified cellulose ion exchangers recently developed by Peterson and Sober (1956) include the carboxylmethyl (CM) diethylaminoethyl (DEAE) and phosphate (P) derivatives These cellulose derivatives have proved to be of unusually high effectiveness for the fractiona tion of proteins especially the plasma proteins and they have a much greater binding capacity for proteins than any of the other ion exchange adsorbents

The application of all of these ion-exchange systems for the separa tions of proteins and especially the blood proteins has recently been reviewed by Sober and Peterson (1957) More recent studies of Sober *et al* (1956) and of Fahey and his co workers (Fahey *et al* 1958 Fahey and Horbett 1959) have made available very valuable chroma tographic fractionation methods for many of the plasma proteins Sober *et al* (1956) used DEAE cellulose and loads as high as 170 mg of protein per gram of adsorbent could be chromatographed with good resolution although the best resolution was with smaller loads Gradient elution was used and it was found that adsorption of a given plasma protein could be reduced in at least three ways (1) by reduction of the pH thus decreasing the anionic character of the plasma protein (2) by increase of the pH thus decreasing the cationic character of the DEAE cellulose adsorbent and (3) by increase in the ionic strength thus decreasing the electrostatic attraction between protein and adsorbent The plasma proteins with isoelectric points for the most part ranging from pH 3 to 8 were applied to the DEAE cellulose column at pH 7 0 and 0 00925 ionic strength (0 005 M total phosphate) Various elution gradients were employed using the fol lowing solutions 0 005 M sodium phosphate buffer pH 7 0 0 02 M sodium phosphate buffer pH 5 9 0 05 M NaH_2PO_4 0 02 M NaCl + 0 05 M NaH_2PO_4 0 05 M NaCl + 0 05 M NaH_2PO_4 0 1 M NaCl + 0 05 M NaH_2PO_4 0 5 M NaCl + 0 1 M NaH_2PO_4 In their latest review Sober and Peterson (1959) apply the proteins to the column at

pH 8 6 in tris * phosphate buffer 0 005 M in phosphate and use a concave gradient from this buffer to 0 5 M tris H_2PO_4 using about 3 4 liters solvent for a 2 gm load of protein on 25 gm of DEAE cellulose The gradient is obtained by the use of a varigrad mixture device as described by Peterson and Sober (1959) The gradient at the end of the elution approaches a pH just under 4 and an ionic strength near 0 5 Fahey *et al* (1958) use a gradient from 0 01 M phosphate at pH 8 0 to 0 30 M phosphate at pH 4 5 (using sodium salts) The ionic strength thus changes from about 0 03 to 0 30

The studies using DEAE cellulose and the plasma proteins have indicated that the isoelectric point of the protein is certainly one of the major factors involved in determining its elution characteristics This is shown nicely by Sober *et al* (1956) in their study of the electrophoretic patterns of various chromatographic fractions This study indicated some definite differences however and there was not an absolute correspondence between the elution volume and the electrophoretic mobility (at pH 8 6 and 0 1 ionic strength) of the various plasma proteins

Another type of modified ion exchange adsorbent has been used by Isliker (1953) and by Isliker and Strauss (1959) for the purification of plasma antibodies By treating a dried carboxylated resin (fine mesh Amberlite IRC 50 called XE 64) with thionyl chloride about 92 per cent of the carboxyl groups of the resin could be converted to acid chlorides Reaction of dried resins with various dilutions of thionyl chloride in chloroform at temperatures near the boiling point of chloroform allowed the preparation of other acid chloride resin derivatives with fewer carboxyl groups substituted One such preparation used by Isliker had 11 per cent of the groups in the acyl chloride form The various resins were then reacted with protein antigens (serum albumin influenza virus and red blood cell stroma) to form chemically modified resin with the protein or stroma chemically linked to the resin probably by amide linkages The two types of resins (92 per cent and 11 per cent conversion to acid chloride) then were found to yield two types of chemically linked materials one thought to represent a thinner layer of antigen (in the 92 per cent conversion) as compared with the other (the 11 per cent conversion) The resins prepared in this way strongly adsorb the specific antibodies (horse anti serum albumin influenza antibody and the human isoagglutinins) from

* Tris is the abbreviation for tris(hydroxymethyl) aminomethane It has a pK of about 8 to 9 (much dependent upon ionic strength and temperature)

serum Difficulties in the elution of the antibodies from the antigen resin complex made quantitative yields of antibody impossible but the method was applied to obtain highly specific antibody in yields of up to 40 per cent

Another type of antigen resin complex was obtained by the treatment of erythrocyte stroma formaldehyde and a strongly basic resin (Amberlite IRA-401) in a sodium tetraborate buffer Further use of such highly specific modified resins may be of value in the isolation of trace components The gentle modification of the antigen to decrease the high energy of binding between antigen and antibody may allow better yields of antibody upon elution

ELECTROPHORETIC FRACTIONATION METHODS

The use of the conventional free electrophoresis method for the isolation of fractions of plasma proteins was introduced shortly after the introduction of the modern Tiselius procedure for analytical electrophoretic studies Even under ideal conditions this method allows the isolation of only a part of the two protein components (those of highest and lowest mobilities) the remainder of the solution being mixtures of the other components and the other part of the components of highest and lowest mobility (Longsworth 1959) It has also been difficult to scale up the procedure in order to use more than about 11 ml of starting solution since larger cells usually have a decreased ability to dissipate the ohmic heating of the solution There is also considerable overlapping of the various electrophoretic components caused by diffusion by the heterogeneity of each of the electrophoretically defined components by convection due to thermal effects and by other boundary anomalies It is thus impossible to obtain good yields of any of the components of serum or plasma without considerable contamination with components of nearby mobilities

These complications have led to the introduction of other types of electrophoretic separations The method of zonal electrophoresis has shown much promise and a wide variety of apparatus has been developed within the past ten years Reviews by Svensson (1948) Tiselius and Flodin (1953) Wunderly (1959) Kunkel and Trautman (1959) Bier (1959) and Wieland (1959) discuss most of the methods which have been introduced In zonal electrophoresis the original mixture of components to be separated is placed in a narrow zone with an

pH 8 6 in tris * phosphate buffer 0 005 M in phosphate and use a concave gradient from this buffer to 0 5 M tris H_2PO_4 using about 3 4 liters solvent for a 2 gm load of protein on 25 gm of DEAE cellulose The gradient is obtained by the use of a varigrad mixture device as described by Peterson and Sober (1959) The gradient at the end of the elution approaches a pH just under 4, and an ionic strength near 0 5 Fahey *et al* (1958) use a gradient from 0 01 M phosphate at pH 8 0 to 0 30 M phosphate at pH 4 5 (using sodium salts) The ionic strength thus changes from about 0 03 to 0 30

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* Tris is the abbreviation for tris(hydroxymethyl) aminomethane It has a pK of about 8 to 9 (much dependent upon ionic strength and temperature)

Various continuous paper electrophoresis separations have been de scribed and rates up to 10 ml per hour have been achieved However the degree of separation between electrophoretic components by con tinuous electrophoresis is not comparable with that achieved by many other types of fractionation methods and Wunderly (1959) summa rizes his discussion of this problem with the statement that even continuous electrophoresis has not yet achieved a satisfactory separa tion of the globulin subfractions in serum Further applications have also demonstrated that a considerable expenditure of work is involved in a technique which permits only micropreparative separations to be made Likewise Bier (1959) states that there are still merely a few proteins or enzymes that are prepared by electrophoresis in pref erence to other methods However the ease of use of this method makes the method useful for certain problems and Bier (1959) sug gests that the amount of work that went into various salt and solvent fractionation schemes is out of all proportion to the effort put into the development of preparative electrophoretic methods although a satisfactory electrophoretic technique would in principle be the ideal solution to all preparative problems

The most extensive study of large scale equipment for zonal elec trophoretic separations has probably been carried out by Porath This work has recently been briefly reviewed by Tiselius (1958) As much as 50 gm of protein have been separated in a single operation using cellulose powder or better an acid-ethanol treated cellulose powder (Flodin and Kupke 1956) in a vertical column and extracting by elu tion the separated proteins from the column

A method showing a great deal of resolution of the plasma proteins has been introduced by Smithies (1955) The procedure uses a high concentration starch gel (15 per cent soluble potato starch in a weak borate buffer) Difficulties in the standardization of the procedure are caused by small variations in the type of starch used and by variation in the potential gradient over different parts of the gel (probably due to the low ionic strength of the buffer) Samples of α_1 globulin α_2 globulin and β globulin are found to show considerable separa tion in this type of electrophoresis but the reasons for this increased resolution are not entirely clear The method has so far been used primarily as an analytical tool but it should be useful as a prepara tive method in the future

The method of immunoelectrophoresis introduced by Grabar and Williams (1955) has also been a most useful analytical tool Here a

electrolyte medium (usually buffered) on either side and connected with suitable electrode vessels through this electrolyte medium Extensive mixing of various zones is prevented by means of a stabilizing density gradient (containing some *non-electrolyte*) or by means of various types of porous supporting media Supporting media which have been successfully used with protein systems are granular starch glass beads powdered cellulose cellulose acetate polyvinyl chloride resin paper various sponges (sponge rubber for example) agar gels and starch gels All of these media induce some electroosmotic flow of the electrolyte solution but with the best materials this flow is minimal The various supporting media also cause some adsorption of certain types of components and may prevent or hinder the motion of large particles or molecules by their restricted pore size

Each of these supporting materials can be used in trays horizontal or vertical tubes and with or without cooling devices Paper can be used without any support and can be hung in various ways Nearly all of the possible combinations of media and apparatus have been proposed The supporting media can be mechanically separated into various zones or the proteins can be extracted from the media by means of a buffer flow in the opposite direction to the electrophoretic migration (Porath *et al* 1958) Most materials can be used either in continuous flow systems or with a given initial charge of solution

With this great array of methods to choose from it is very difficult to briefly discuss the electrophoretic fractionation method The following discussion is concentrated on a few methods (not necessarily the most useful) that have been extensively used for the fractionation of plasma proteins

Paper has been widely used because of the somewhat simpler apparatus required for its application The most easily available method in most laboratories is to take an apparatus designed for analytical paper electrophoresis and use either several of the usual size strips or one large strip Heavy papers (Schleicher and Schull No 589 green ribbon or Whatman #31 for example) may replace the usual type or multiple thicknesses of the conventional paper may be used By staining a small part of the strip (or transfer of the strip) the papers can be cut into appropriate segments and the protein removed by centrifugation or elution In this way 1 to 5 ml of serum can be electrophoretically fractionated in a single batch The fractions obtained in this way seldom if ever represent pure electrophoretic components but a high degree of fractionation can be achieved (Wunderly 1959)

Various continuous paper electrophoresis separations have been de scribed and rates up to 10 ml per hour have been achieved However the degree of separation between electrophoretic components by con tinuous electrophoresis is not comparable with that achieved by many other types of fractionation methods and Wunderly (1959) summa rizes his discussion of this problem with the statement that even continuous electrophoresis has not yet achieved a satisfactory separa tion of the globulin subfractions in serum Further applications have also demonstrated that a considerable expenditure of work is involved in a technique which permits only micropreparative separations to be made Likewise Bier (1959) states that there are still merely a few proteins or enzymes that are prepared by electrophoresis in pref erence to other methods However the ease of use of this method makes the method useful for certain problems and Bier (1959) sug gests that the amount of work that went into various salt and solvent fractionation schemes is out of all proportion to the effort put into the development of preparative electrophoretic methods although a satisfactory electrophoretic technique would in principle be the ideal solution to all preparative problems

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zonal electrophoretic separation is carried out in agar filled trays and then an antiserum is introduced into a trough running parallel to the direction of electrophoretic development and a few centimeters dis tant from the point of application of the sample Diffusion allows the mixing of these reagents and a large number of opaque bands develop each indicating the presence of a certain protein component At least 18 different components have been detected in normal human serum using this procedure This procedure cannot be used as a pre parative method but it is of great usefulness in establishing the homogeneity of serum fractions

Preparative electrophoresis without a supporting medium while the oretically the most attractive approach has not been developed into highly useful procedures The various approaches to this problem have been reviewed by Bier (1959) who classified the various methods as density gradient electrophoresis electrodecantation electrophoresis convection and forced flow electrophoresis

Completely satisfactory density gradient electrophoresis has not yet been achieved However a method of Kolin (1954 1955) utilizing a sucrose stabilized pH gradient of low ionic strength has permitted a very rapid separation of protein mixtures into bands of proteins sep arating by virtue of the isoelectric points of the components Svensson *et al* (1957) and Berg and Beeler (1958) used as much as 300 mg of serum protein and obtained reasonably good resolution using a buf fered sucrose gradient

The electrodecantation method allows the concentration of a col loidal (and hence slowly diffusing) material at an electrode By holding the pH of the solution at a value near the isoelectric point of the most alkaline component (γ globulin in the case of the serum pro teins) it is possible to separate fair yields of this component as a top solution with the other components concentrating at the bottom Polson (1953) has used this method for the preparation of γ globulin and certain viruses

The method of electrophoresis-convection introduced by Kirkwood has been applied to the fractionation of a number of plasma protein materials The protein is subjected to horizontal transport by electro phoresis using a long narrow channel with the long axis vertical The protein components concentrate at a surface membrane and the increased concentration causes an increase in density with the com ponents of highest mobilities concentrating a reservoir at the bottom of the apparatus the top reservoir containing enriched amounts of

the slowest migrating component A series of studies of whole serum or of plasma fractions have shown the usefulness of this technique and some seventeen references to work of Kirkwood Cann and Timasheff utilizing this technique for the fractionation of serum or plasma fractions with particular emphasis on the antibody distributions will be found in the review of Bier (1959)

The method of forced flow electrophoresis was introduced by Bier and is a dynamic method where protein solution is made to flow into a cell of somewhat the same characteristics as the electrophoretic cell of Kirkwood Two solutions emerge one solution being enriched with the components of highest mobilities and the other solution containing less of these components The method has been used for the concentration of γ globulin from serum and for the preparation of certain enzymes and bacterial polysaccharides The method is described by Bier (1959) and references to other work may be found in this review

ULTRACENTRIFUGAL FRACTIONATION METHODS

Use of ultracentrifugal methods for the fractionation of protein solutions has been made in several ways The determination of the amount of sedimentable material has been used as a method for distinguishing between protein bound and protein free concentrations of low molecular weight materials The experiments of Chanutin *et al* (1942) on the calcium-casein interaction illustrate this type of experiment The ultracentrifuge has also been used for the separation of the proteins of higher sedimentation constant although this procedure yields only highly purified components after a series of sedimentation procedures each capable of concentrating the faster sedimenting components Use of this procedure has led to claims that certain activities are associated with components of high sedimentation constant For example Pedersen (1945) has estimated that the human isoagglutinins have a sedimentation constant $s_{20,w}$ near 20 S and Pillemer *et al* (1954) assign a sedimentation constant of 27 to properdin Unless such studies show the identity of sedimentation rate as measured by protein and by activity as discussed earlier (p 15) these measurements have little meaning Measurements of this type can be made best in the swinging bucket rotor using a technique like that of Hogeboom and Kuff (1954) or in a separation cell of the type developed by Yphantis

and Waugh (1956b) Yphantis and Waugh (1956a) also discuss the theory of this method

The ultracentrifuge has been more useful for the preparation of various lipoproteins Since the lipid causes the density (or partial specific volume) of the lipoprotein to have values much different from those of ordinary proteins it is possible to separate these components on the basis of their lower densities Two techniques have been used One uses a series of solvents of increasing density and the lipoproteins of density less than the solvent being used in any particular experiment are then concentrated in a top layer In this way lipoproteins of density less than 1 003 between 1 003 and 1 062 and between 1 062 and 1 21 have been obtained by a number of workers A description of a suitable technique for such separations will be found in the paper of deLalla and Gofman (1954) A more powerful fractionation technique uses a density gradient in plastic centrifuge tubes and after ultracentrifugation of these tubes the lipoproteins are concentrated in zones where the density of the lipoprotein is equal to the density of the solvent This technique can use sodium chloride or sodium chloride–potassium bromide gradients which slowly change due to diffusion as described by Mannick and Oncley (1954) and by Oncley *et al* (1957) or it may consist of equilibrium gradients usually using cesium chloride of the type introduced by Meselson *et al* (1957) This later method must be used for lipoproteins of low sedimentation constant and has been especially useful in the study of nucleic acid homogeneity

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PART TWO

Hormones of Pancreatic Origin

CHAPTER III

Insulin-Like Activity and Anti-Insulin Factors in Human Plasma

Albert E Renold, Paul M Beigelman, A F Willebrands, J Groen, Donald B Martin, Yves M Dagenais, Solomon A Berson, Rosalyn S Yalow, Maurice E Krahl *and* Harry N Antoniades

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Introduction

Albert E Renold

Extensive and detailed information is available regarding a large number of chemical and physical properties of insulin extracted from pancreatic tissue Indeed the amino acid composition sequence and structure are known for several insulins of mammalian origin although not as yet for human insulin Despite this apparently sound chemical and physicochemical basis however precise information concerning the synthesis storage secretion and transport of insulin in the mammalian organism is remarkably scant Among the factors responsible for this lack of information inability to measure insulin accurately in blood and in other biological fluids is probably foremost

Analysis of the difficulties to be surmounted in order to develop a satisfactory measurement for insulin in blood must begin with the problem of quantity According to the best available estimates plasma or serum from fasting human subjects contains approximately 100 to 1000 microunits of active insulin per milliliter which means that in order to obtain 1 mg of active insulin between 20 and 200 liters of serum or plasma would be required At present only procedures based on the measurement of biological activity or on immunological techniques are likely to offer both sufficient sensitivity and sufficient specificity for a suitable assay technique The major portion of this chapter will be devoted to the description and evaluation of the procedures which have been proposed as presently or potentially suitable for bioassay or immunoassay for the hormone

Although several animal or tissue preparations are now known to be sufficiently sensitive to insulin to detect quantities of the hormone likely to be present in serum or plasma the parameters of insulin activity which have been used have as a rule been rather nonspecific ones i e most frequently the production of hypoglycemia *in vivo* or of increased glucose uptake *in vitro* These parameters are known to be affected by a number of factors other than insulin — such as by other hormones — and as a result the activity measured has never been insulin activity but *net insulin like activity* A section of this chapter will be concerned with the present state of our knowledge concerning factors other than insulin which are known to affect either insulin action or the measurement of net insulin like activity

It would obviously be desirable to purify plasma or serum insulin before measuring it In attempting to devise means for the purification of insulin in serum or plasma it would seem essential to be fully informed with regard to the chemical and physicochemical state of insulin in serum or plasma Since the vast and detailed body of information concerning insulin has been accumulated for insulin extracted from pancreatic tissue by drastic procedures it is entirely conceivable that it is applicable only in part to insulin physiologically secreted into and carried in the blood stream Indeed even the biological properties of plasma insulin need not be identical with those of insulin extracted from the pancreas Accordingly it seemed important to include in this chapter a discussion of the present state of knowledge concerning the state of insulin in the circulation despite the evident limitations of this knowledge

Finally it should be noted that the recent demonstration of the widespread occurrence of insulin reactive antibodies in insulin treated individuals has severely shaken the previous concept of the low antigenic activity of the hormone It is likely that future chapters concerned with insulin in plasma will give increasing prominence to the consideration of the immunological reactions elicited by exogenous and conceivably endogenous insulin

Insulin Bioassays in Vivo*

Paul M Beigelman

For a number of years efforts have been made to devise an effective simple sensitive and reproducible method for assay of insulin and insulin like substances utilizing *in vivo* techniques Various *in vivo* preparations have been investigated including intact mice (Beigelman 1958 Goetz and Egdahl 1958) hypophysectomized adrenodemedullated rats (Gellhorn *et al* 1941 Kosaka *et al* 1957) hypophysectomized alloxanized mice (Yenerman *et al* 1953 Goetz *et al* 1954 Anderson *et al* 1957) and hypophysectomized alloxanized rats (Bornstein 1950 Beigelman *et al* 1956a) • Several variations have been described in each instance and will be briefly discussed It should be emphasized that only assays suitable for the measurement of the very small quantities of insulin expected in plasma will be discussed here Bioassays suitable for the measurement of larger quantities have of course been in satisfactory use for many years

DESCRIPTION OF SOME PROCEDURES WHICH HAVE BEEN REPORTED

Intact Mice

Methods have been recently reported for assay of insulin and insulin like substances utilizing blood glucose decrement of intact mice and they are mentioned first since in the opinion of the author they offer some promise of being the most practical and yet adequate of the *in vivo* techniques Insulin concentrations in the range 2 5 to 10 milliunits have been measured by this method by Goetz and Egdahl (1958) and as adapted by Beigelman (1958) the method has been shown to be sensitive to 1 milliunit of insulin and to have an index of precision (λ) of 0 52 These figures were derived from data obtained by determining blood glucose decrements 1 hour following intraperitoneal injection of graded doses of insulin into 20 to 30 gm intact mice anesthetized with pentobarbital

Advantages of this method include elimination of extensive or

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difficult preparation of the animals and relative simplicity of technique It has been successfully employed for the determination of insulin like activity of serum protein fractions (Beigelman 1958) Probably an important precaution required in performing this and other techniques involving assay of highly diluted insulin is that the insulin be diluted in protein-containing rather than simple electrolyte or aqueous solutions

Hypophysectomized adrenodemedullated Rats

Gellhorn *et al* (1941) performed insulin assays by employing hypophysectomized adrenodemedullated rats Insulin or blood was injected intraperitoneally into these preparations the blood glucose decrement being measured over the ensuing 1 hour period Minimal sensitivity with this technique was reported to be 0 3 milliunit and insulin like activity so measured could be recovered when insulin was added to whole blood One milliliter of human blood secured 2½ hours postprandially caused a slight but consistent fall in blood glucose when injected into hypophysectomized adrenodemedullated rats This was not observed in normal intact rats Gellhorn *et al* (1941) on the basis of these data estimated the normal human blood insulin like concentration to be about 0 2 milliunit per milliliter

Kosaka *et al* (1957) recently reported a similar technique of insulin assay utilizing hypophysectomized adrenodemedullated rats These investigators noted that blood glucose remains stable for 2 hours following a 3 hour period of fasting in these animals thus providing a suitable control basis for the measurement of insulin like activity The smallest quantity of insulin measurable with this technique was 0 2 milliunit Insulin like activity of peripheral blood plasma from fasting human beings and dogs was too low to be estimated Normal human plasma insulin like activity 90 to 120 minutes after a meal was approximately 0 3 milliunit per milliliter In dogs insulin like activity 105 minutes following intravenous administration of 20 gm of glucose was 0 3 to 0 4 milliunit per milliliter

Hypophysectomized alloxanized adrenalectomized (or adrenodemedullated) Rats

Anderson *et al* (1947) reported sensitivity of 125 microunits (0 125 milliunit) of insulin in adrenodemedullated hypophysectomized alloxanized rats when the blood glucose response was measured 1 hour following the intravenous injection of insulin 30 minutes after glucose

in hypophysectomized alloxanized mice following gavage with the polysaccharide dextrin This technique appeared to combine great sensitivity and precision but subsequent studies from the same laboratory did not fully confirm the results initially obtained The principal difficulty also encountered by Goetz *et al* (1954) was the poor predictability of the blood glucose response to control saline injection Goetz *et al* (1954) devised a modification of this technique in which the initial 1 hour blood glucose increase following gavage with the polysaccharide dextrin was measured The diminution of this initial 1 hour increase could be related to graded doses of insulin or to insulin like activity The method was sensitive to approximately 1 to 2 milliunits of insulin but not sufficiently sensitive to detect insulin like activity in whole serum or plasma However the technique was effectively employed in the study of serum protein fractions These mouse preparations however were difficult to obtain presented a high and unpredictable mortality rate and yielded a relatively insensitive and imprecise assay

Hypophysectomized alloxanized Rats Receiving Carbohydrate Gavage

Hypophysectomized alloxanized rats have also been employed for assay purposes utilizing the initial 1 hour blood glucose increment following gavage with dextrin as the measured parameter (Beigelman *et al* 1956b) Diminutions in blood glucose increment could be related to insulin or insulin like factors administered by the intra peritoneal route simultaneously with the dextrin gavage Rigorous control of many variables was required Thus it was necessary to maintain a constant warm environment the temperature remaining at 27 to 28°C Special cubicles were constructed for the rats effecting separation and almost complete suppression of physical activity during the course of the procedure Since dextrin administered in small quantities elicited an unpredictable blood glucose elevation whereas excessive quantities yielded insensitive preparations a standard dosage of 300 mg of dextrin was selected Rats with initial blood glucose levels of less than 300 mg per hundred milliliters appeared to be more satisfactory By rigid adherence to these requirements diminution in blood glucose increment could be directly related to dosage of insulin and a relatively insensitive but reproducible technique of insulin assay was obtained The method was sensitive to 2 5 milliunits of insulin and the index of precision (λ) was 0 305 This sensitivity was not sufficient to detect insulin like activity in whole serum or plasma

but the technique was satisfactorily utilized for the detection of insulin like activity in plasma protein fractions The mortality rate of the rats was considerably less than that of the hypophysectomized alloxanized mice

TABLE I *In Vivo* Bioassays for Insulin like Activity

A thor	Animal p epa ti	Pa am t f insulin acti ity	Sensiti ity (n milli nits)	*Index f* p eci i (λ)
Goetz and Egdahl (1958)	Inta t mo e	Blood gl c e decreme t	2 5	N t repo ted
Belg lman (1958)	I ta t mo e	Blood gl co e *d cr m t 1 hr* afte i p l je ti a e the ia with p tobarbital	1 0	0 52
Gellhorn, Feldman and Allen (1941)	Hypox. adr node m d llated t	Blood gl cose d c m t 1 h afte i l je tio of ins li	0 3	Not p ted
Anders n, Li dner and Sutton (1947)	Adr node medull ted H. A.†	Blood gl cose re ponse to i.v i li ov r ½ hr pe iod i l lin ½ hr after gl co e gavag	0 125	Not r po ted
Ko aka, Id a d Kuz ya (1957)	Hypox. adr node m d ll ted rat	Blood gl cos d eme t	0 2	N t epo t d
Bornst i (1950)	Adre H. A.† rat	Blood gl co e decrement	0 05	Not ported
Anders n, Wherry B tes a d *Cornfield* (1957)	H. A.† mou	Sub ta eou i jec *tio of gl co e 1 hr* b fo gl s de m t me ed		
		Blood gl de eme t 20 mins after i v i j cti of ins lin	0 25‡ 0 25§	0 78 0 68
		Blood gl c d m t 30 mi aft i p i je ti f ins li	0 5‡ 0 5§	0 48 0 37
Go t , B ig lma nd Th rn (1954)	H. A.† mo se	Dim i h d i e me t f blood gl o 1 hr aft r ga ag *with d trl*	1 0 2 0	Not d te mined
Beigelman, Go tz Anto iad and Tho (1956b)	H. A.† Rat	Dimi i h d i c e m t f blood gl co e 1 hr after g ge with dextri	2 5	0 305

Adrex Adrenal tomi d
† H A. Hypophy ectomi ed all ani ed
‡ Fi t us
§ Second and ub eque t

DISCUSSION

A number of *in vivo* techniques using intact mice or various com binations of hypophysectomy alloxanization and adrenalectomy in

in hypophysectomized alloxanized mice following gavage with the polysaccharide dextrin This technique appeared to combine great sensitivity and precision but subsequent studies from the same laboratory did not fully confirm the results initially obtained The principal difficulty also encountered by Goetz *et al* (1954) was the poor predictability of the blood glucose response to control saline injection Goetz *et al* (1954) devised a modification of this technique in which the initial 1 hour blood glucose increase following gavage with the polysaccharide dextrin was measured The diminution of this initial 1 hour increase could be related to graded doses of insulin or to insulin like activity The method was sensitive to approximately 1 to 2 milliunits of insulin but not sufficiently sensitive to detect insulin like activity in whole serum or plasma However the technique was effectively employed in the study of serum protein fractions These mouse preparations however were difficult to obtain presented a high and unpredictable mortality rate and yielded a relatively insensitive and imprecise assay

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III Nine bulls with considerable disturbance in spermatogenesis Six were Swedish Red and three Friesian

IV Four bulls with combined disturbance in spermatogenesis and ability to serve

The results obtained so far, show that the greatest frequency of sterility appears in the first year of service, i e at the age of 18–30 months This and other facts (the results of investigations on one egg twins etc) show definitely that in most cases sterility in young bulls is due to an inborn tendency towards sexual disturbances as a result of an inherited weak endocrine constitution There are, of course, also other causes of infertility in young bulls

Only bulls in which the infertility, in all probability was caused by inherited factors are included in this investigation This report includes only bulls younger than 36 months

After having been in regular service the bulls appeared either to be unable to serve or to produce infertile sperm and were therefore sent to the Veterinary College where they were kept for several months During this period they were subjected to thorough clinical examination and after slaughter the endocrine and sexual organs were examined histologically The results of these researches will be published later by Lagerlof and co workers

Methods

The urine samples were taken daily between 8 a m and 10 a m and analysed during the same day

As shown by Koch (1942) and others the urinary excretion of steroids in man may vary from day to day To avoid such variations the urine samples were taken for eight ten or more consecutive days and the mean values of the determinations were used

Twenty to 50 urine samples were taken from some bulls and examination of the results showed that there was some constancy in the values of steroids excreted The average excretion of eight to ten consecutive samples seems to be typical for the animal concerned

As it was not possible to collect 24 hour samples the specific gravity of the urine was taken into consideration in calculating the neutral steroid content Dingemanse *et al* (1937)

rats and mice have been utilized for the assay of insulin and insulin like material These methods indicate that the normal serum or plasma insulin like activity is in the range of 0 1 to 0 3 milliunit per milliliter

In the opinion of the author the procedure employing intact mice is the simplest yet appears to demonstrate sensitivity and precision comparable to that obtained with the other more complex *in vivo*

TABLE II Level of Insulin like Activity in Pancreatic Vein Plasma in Anesthetized Dogs

Treatment	No exp	Period of collection (minutes)	Blood sugar range (mg %)	Hematocrit	Insulin microunits per ml of plasma
Control saline only	6	43	72 49	43	65 ± 19* (26 - 137)
Glucose 50 gm	4	63	228 701	47	302 ± 45 (207 385)
Growth hormone 50 - 100 mg	4	38	67 34	59	96 ± 19 (48 - 125)
Tolbutamide 1 0 gm	6	46	90 - 25	48	136 + 21 (61 - 185)

*+ S E and range

methods However all *in vivo* methods appear to be less sensitive and more difficult to perform than certain of the newer *in vitro* techniques employing rat diaphragm and adipose tissue Nonetheless it is important to realize that under certain circumstances various *in vivo* preparations may respond to insulin like or anti insulin factors which may not affect *in vitro* preparations Also factitious insulin like effects may be observed *in vitro* and not *in vivo* As long as the measurement of insulin in serum or plasma is dependent upon the use of bioassay techniques the combined use of *in vitro* and *in vivo* procedures to identify the nature of the activity measured remains desirable Some of the information discussed in this section has been gathered in Table I

Recently Anderson *et al* (1959) have reported values obtained with the method of Anderson *et al* (1957) applied to pancreatic vein plasma fractions obtained by the method of Bates (1958) The author is indebted to Dr Anderson for permission to include Table II which summarizes the material presented by her at the Annual Meeting of

tion The possibility that substances other than insulin may be present in the serum and may possess insulin like activity is not excluded There is evidence however that the substance responsible for the insulin activity of serum is protein in nature (cerebrospinal fluid and the ultrafiltrate of normal human serum exert no activity) (Willebrands *et al* 1958b) It has been suggested that part of the insulin like effect of serum is due to growth hormone or to a non specific effect of the serum proteins Ottaway (1953) reported that growth hormone in rather high concentrations enhances glucose utilization of diaphragm tissue Randle (1954a) found high serum insulin values in acromegaly and low ones in hypopituitarism (Randle 1954b) However Randle and Whitney (1957) recently reported that the effect of growth hormone depends on the composition of the buffer in the medium In phosphate buffer growth hormone exerted insulin like activity in concentrations of 10 to 100 μg per milliliter while in Gey and Gey (1936) buffer (a bicarbonate buffer frequently used for insulin determinations) no effect was observed (Park *et al* 1952) Bornstein and Park (1953) who reported that bovine albumin has an insulin like effect suggest that the insulin effect of serum is nonspecific The authors have found that some commercial serum protein fractions obtained by precipitation techniques often enhance or depress the glucose uptake of the diaphragm muscle when added to the incubation medium However these effects often decreased considerably after dialysis of the protein fractions or treatment with cysteine or glutathione (Willebrands and Groen 1958) Therefore the depressing effects of these protein fractions may well be due to the preparation procedure whereas the stimulating effects may be due to insulin from the original serum (Taylor and Randle 1959 Randle and Taylor 1958a) Albumin fractions obtained by column electrophoresis of normal serum exhibit no insulin activity (Randle and Taylor 1958a Bolinger *et al* 1958a 1959) Thus arguments for the presence of natural substances with insulin like activity — other than insulin — in serum do not seem convincing On the other hand the following observations suggest that the insulin activity of serum is due primarily to insulin itself and not to nonspecific factors

(1) Serum from dogs or cats taken a few days after total pancreatectomy does not stimulate glucose utilization by rat diaphragm (Groen *et al* 1952 Vallance Owen and Lukens 1957)

(2) The activity of crystalline insulin is inhibited by cysteine or glutathione so is the serum insulin like activity when the serum is

Section 2

Insulin Bioassays in Vitro Using Isolated Rat Diaphragm*

A F Willebrands and *J Groen*

PRINCIPLE

The *in vitro* method for the determination of serum† insulin using the surviving rat diaphragm is based on the fundamental observation of Gemmill (Gemmill 1940 Gemmill 1941 Gemmill and Hamman 1941) who showed that insulin increases the glucose uptake and glyco gen synthesis of this muscle preparation This *in vitro* effect of insulin is obtained even when the concentration of the hormone in the incu bation medium is very small the effect of 0 1 milliunit of insulin or less can be demonstrated easily (Willebrands *et al* 1950) Serum of normal human beings rats and dogs also enhances glucose utilization and glycogen synthesis by the diaphragm preparation (Tuerkischer and Wertheimer 1948 Kamminga *et al* 1950 Groen *et al* 1952) As is discussed later the insulin like activity of serum is likely to represent true insulin activity An estimation of serum insulin concentration or better of effective serum insulin concentration can therefore be made by comparing the insulin activity of serum with the activity which crystalline insulin exerts on the isolated rat diaphragm under the same conditions The insulin concentration thus determined is expressed in terms of milliunits (1 milliunit = 10^{-3} unit) or microunits (1 microunit = 10^{-6} unit) of insulin per milliliter of serum This method of insulin determination is highly sensitive but not very accurate its specificity is not as yet fully proven and the presence of insulin antagonists may interfere with the insulin determination It is the purpose of this review to consider in greater detail some aspects of insulin determination and to review the progress made since our last survey (Willebrands and Groen 1954)

SPECIFICITY

The specificity of the rat diaphragm method is under discussion since whole serum without modification is used for the determina

* Research supported by the American Diabetes Association the Netherlands Society of Diabetes and Organon Laboratories Inc

† As no difference between serum and plasma insulin like activity has been found only serum will be mentioned

(1956a) reported that plasma insulin can be absorbed on a column of IRC 50 or Dowex 50 resin and can be recovered by elution with dilute citric acid solutions This procedure may provide a simple and useful method for separating relatively pure serum insulin

Haavaldsen and Walaas (1957) have reported that dialysates from serum or plasma stimulate glucose uptake by diaphragm tissue thereby casting some doubt on the specificity of the diaphragm method since pure insulin was not removed by dialysis under the same conditions However this finding could not be confirmed by one of the present writers (Willebrands) Several recent reports have also appeared suggesting insulin like activity of certain amino acids in the diaphragm test (Bolinger Walaas 1958) However there appears to be disagreement as to the activity of some of the amino acids used and Willebrands was unable to confirm some of the observations reported In most instances the effects of amino acids on glucose uptake were small and occurred at concentrations unlikely to be found in diluted serum Furthermore Bolinger noticed that amino acids which promoted glucose uptake did not appear to increase diaphragm glycogen in striking contrast to insulin and also to serum

ANTAGONISTS

The results of the diaphragm assay are influenced by the presence of insulin antagonists From a theoretical point of view the different types of antagonists must be considered

Hormone Antagonists

Hormones are already known to influence carbohydrate metabolism *in vitro* (Renold *et al* 1956) These include the hormones of the thyroid gland the somatotrophic and adrenocorticotrophic hormones of the pituitary gland and the glucocorticoids epinephrine nor epinephrine and glucagon Wertheimer and Bentor (1953) reported that thyroxine diminished glucose utilization in small concentrations only in the presence of homologous serum and not in bicarbonate buffer The possibility that thyroxine has an anti insulin effect on the diaphragm insulin system has not been investigated

Information about pituitary hormones antagonizing the action of insulin on the rat diaphragm are restricted to growth hormone and to crude anterior pituitary extract Stadie *et al* (1949) reported that

treated with these substances (Groen *et al* 1952 Randle 1954c Vallance Owen and Hurlock 1954)

(3) The stimulating effect of serum such as found with crystalline insulin is greater with hemidiaphragms than with diaphragms that are divided into four or eight parts (Willebrands *et al*, 1951 Liebecq 1957) This observation appears reasonable since insulin is now generally assumed to enhance the penetration of glucose into intact muscle cells (Levine 1958)

(4) The activity of crystalline insulin as well as that of serum diminishes greatly when epinephrine or norepinephrine is added to the incubation medium in small concentration (Groen *et al* 1958 v d Geld *et al* 1958)

(5) In column electrophoresis the mobility of normal human serum insulin is identical to that of crystalline insulin or insulin I^{131} added to serum *in vitro* (Bolinger *et al* 1958a 1959)

(6) Randle and Taylor (1958a) and Wright (1959) have reported that antisera to ox insulin prepared in guinea pigs can prevent the action of ox insulin as well as the insulin like action of ox serum on the isolated rat diaphragm Normal guinea pig serum has no such effect As these antibodies seem to be highly specific these experiments also suggest the presence of insulin in serum

(7) Both crystalline insulin and serum insulin increase glucose utilization by rat diaphragm as well as by mesenteric fat tissue (v d Geld *et al* 1957) and epididymal fat *in vitro* (Martin *et al* 1958)

(8) Insulin activity is greatly diminished or absent during diabetic coma and in severe juvenile diabetes (Groen *et al* 1952 Vallance Owen and Hurlock 1954) it has been found very high in islet cell adenoma of the pancreas (Groen *et al* 1957)

These findings point to the conclusion that insulin is the substance in serum which is capable of stimulating glucose uptake in rat diaphragm although decisive evidence must wait until separation and purification procedures for serum insulin are available Progress in the development of such procedures has been reported by Baird and Bornstein (1957) and by Antoniades (1958) The first named authors prepared extracts of serum with a low protein content and appreciable insulin activity Bioassays carried out on these extracts suggest insulin levels of 1 to 2 milliunits per milliliter in healthy adults after a meal these are values of the same order as those found by the authors and others using whole serum with the diaphragm technique Antoniades (1958) following earlier investigations of Beigelman *et al*

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crude anterior pituitary extract diminishes the increase of glycogen synthesis caused by insulin but that pure growth hormone is not active in this respect

The reports on the effects of steroid compounds are somewhat confusing Verzár and Wenner (1948a) and Leupin and Verzár (1949) showed that the effect of insulin on glycogen synthesis and glucose uptake was diminished by certain steroids in a concentration of 10 to 100 μg per milliliter Bartlett *et al* (1949) confirmed these findings on the effect of steroids on glycogen synthesis However glucose utilization was found not to be affected Stadie and co workers (1951) reported that the addition of 50 μg of 17 hydroxycorticosterone (cortisone) or 11 desoxycorticosterone per milliliter of incubation medium diminishes glycogen synthesis in the diaphragm Candela *et al* (1951) reported that 3 to 7 μg of cortisone per hundred milligrams of diaphragm tissue decreased glucose utilization However Kamminga *et al* (1951) found that the same steroid in a concentration of 25 μg per milliliter only slightly diminished glucose uptake and left unchanged the effect of 0 5 milliunit of insulin per milliliter Field and Stetten (1956) found that 1 μg of cortisol had no anti insulin effect on glycogen formation

In these experiments however the concentrations of steroids in the medium were much higher than in normal or pathological sera It seems improbable that glucose metabolism of the isolated rat diaphragm would be affected by the amounts of glucocorticoids present in normal or pathological human serum

The anti insulin effect of epinephrine on diaphragm tissue has been known from the work of Walaas and Walaas (1950) and Riesser (1947) although the concentrations of epinephrine used by these authors were much higher than those present in normal or pathological sera Groen *et al* (1958) and v d Geld *et al* (1958) disclosed however that very low concentrations of epinephrine inhibit the action of physiological amounts of insulin in the diaphragm assay 10^{-2} μg per milliliter of epinephrine counteracts almost completely the effect of *0 5 milliunit per milliliter of insulin while significant* inhibition still results at concentrations as low as 10^{-4} μg per milliliter Norepinephrine is also active in this respect but in concentrations about one hundred times higher The normal concentration of epinephrine in serum is still under discussion (see Vogt Chapter XVI) but concentrations of a magnitude of 0 1 μg per liter (10^{-4} μg per milliliter) have been reported as a likely normal value (de Valk

and Price 1956 von Euler 1957) Since the serum is diluted 1 10 in the technique recommended by us epinephrine is unlikely to be a disturbing element but in insulin determinations in which sera with high epinephrine levels or undiluted sera are used the reverse might be true (Groen *et al* 1957) High levels of epinephrine occur in the sera of some patients with pheochromocytoma and with hypoglycemia (von Euler and Luft 1956)

According to Candela (1953) glucagon also has an epinephrine like action on diaphragm muscle However Bolinger *et al* (1958b) have been unable to confirm this They found that pure glucagon in concentrations of 0 1 to 10 μg per milliliter did not inhibit the activity of 0 5 milliunit of insulin per milliliter

Insulin binding Antibodies

It seems certain that some cases of insulin resistance must be explained by inactivation of insulin by antibodies (Colwell and Weiger 1956 DeFilippis and Iannaccone 1952) A similar course of events may play a role in many ordinary cases of diabetes It is certainly possible that specific antibodies against insulin interfere with the diaphragm assay for as has already been mentioned Randle and Taylor (1958a) as well as Wright (1959) reported that guinea pig antisera against bovine insulin abolished the action of bovine insulin and also the insulin like action of bovine serum in the diaphragm bioassay Therefore the possibility that patients develop antibodies against exogenous insulin and that these antibodies interfere with the assay cannot be excluded Marsh and Haugaard (1952) found that sera from several insulin resistant diabetics interfered with the combination of insulin with the rat diaphragm

Other Insulin Antagonists of Unknown Nature

According to Bornstein and Park (1953) glucose utilization of normal diaphragm in a medium containing serum of alloxan diabetic rats is lower than in a medium containing normal rat serum Comparison of the effect of normal rat serum with that of the serum of diabetic rats previously hypophysectomized or adrenalectomized disclosed no difference in glucose uptake These authors assume that the blood of alloxan diabetic rats contains an insulin inhibitor formed by the interaction of two factors one originating from the pituitary and one from the adrenal cortex (possibly growth hormone and a glucocorticoid) This inhibitor seems to be present in the lipoprotein

fraction of the serum (Bornstein 1953) No further progress in the study of this complicated antagonistic mechanism has been reported to date Vallance Owen and Lukens (1957) came to similar conclusions after a study of the insulin like activity of depancreatized depancreatized hypophysectomized and depancreatized adrenalectomized cats

Evidence that humoral insulin antagonists interfering with glucose uptake by rat diaphragm may also occur in the serum of human diabetic patients has been reported by Vallance Owen *et al* (1955) by Field and Stetten (1956) and by Baird and Bornstein (1957) The second named authors found that in most cases of diabetic acidosis the serum exhibited insulin inhibiting properties which disappeared after recovery The substance has not yet been identified but it is not dialyzable Its electrophoretic mobility is that of α_1 globulins it is not a lipoprotein and it is inactivated by heating Baird and Bornstein (1957) in an attempt to purify insulin like activity of serum and to separate it from possible insulin antagonists were able to prepare fractions from the plasma of diabetic patients which exerted insulin antagonistic activity in the diaphragm bioassay Vallance Owen *et al* (1955) found no insulin like activity in the plasma of uncontrolled but nonketotic diabetic patients When insulin was added to the plasma of these patients *in vitro* it was not recovered in the diaphragm test Willebrands (1958) discovered in several cases of diabetic coma that the serum contained only very small amounts or no insulin activity and that the activity of added insulin was abolished in varying degrees

We may summarize these considerations as follows Of the well known hormones it is likely that only epinephrine can act as an insulin antagonist in the diaphragm test i e in cases with high serum epinephrine levels or when undiluted serum is used for insulin determination Further possible antagonists are insulin binding antibodies such as might be present in cases of insulin resistance while *certain humoral antagonists of as yet unknown structure* seem to be present in certain forms of diabetes or at certain stages of the disease especially during diabetic acidosis Whether an increased serum content of epinephrine could also be the cause of the antagonistic activity in this condition is at present under investigation

As long as the role of these factors has not been fully investigated it remains difficult or even impossible to estimate the true insulin level of serum and it may be preferable to regard the test as a measure of

the result of insulin and anti insulin factors in terms of effective serum insulin On the other hand the diaphragm technique may be used as a tool to demonstrate the presence and estimate the strength of the anti insulin factors

The antagonist described by Field and Stetten (1956) has been further investigated but its nature has not yet been elucidated (Field 1958) With a more sensitive test procedure the presence of an insulin inhibitor could also be demonstrated in the serum of several patients with newly discovered diabetes before insulin therapy was started (Field 1958) It is not clear whether this inhibitor is identical with the one which occurs in diabetic acidosis Vallance Owen *et al* (1958a b) reported that the insulin inhibiting substance they had found in plasma from uncontrolled insulin requiring diabetics is associated with but not identical with the serum albumin fraction Even the albumin fraction of normal serum was found to exhibit some insulin inhibiting activity It has to be kept in mind that these experiments were carried out with rather high concentrations of albumin (3% to 5%) in the incubation medium This is perhaps the reason why Willebrands using lower concentrations was unable to detect insulin inhibiting activity in the plasma albumin fractions of similar patients

SYNERGISTS

Synergists in this case refers to substances with insulin like action which do not occur naturally

Manchester *et al* (1958) reported that salicylates exert insulin like action in the diaphragm test 5×10^{-3} mole of salicylate equals about 10^{-4} microunits per milliliter of insulin activity

According to Rafaelsen (1958) carbutamide tolbutamide and phenetyldiguanide increase glucose uptake of the diaphragm when added to the incubation medium However these findings have not been confirmed by other workers (Field and Woodson 1956 Ashmore *et al* 1957)

The finding of Manchester *et al* (1958) that salicylate exerts insulin like activity in so far as it stimulates glucose uptake by diaphragm could be confirmed (Willebrands) The literature on the influence of the oral antidiabetic drugs on glucose uptake by the rat diaphragm remains conflicting (Willebrands Bolinger Clarke *et al* 1956 Cahill *et al* 1957 Recant and Fischer 1957 Lundbaek *et al* 1958 Leijnse *et al* 1959) with the exception that phenethyl biguanide (D B I)

appears to exert a marked insulin like effect with regard to glucose uptake (Willebrands Bolinger Williams *et al* 1957) although not with regard to glycogen deposition (Bolinger Lundbaek *et al* 1958)

SENSITIVITY

The sensitivity of the rat diaphragm to insulin varies widely owing to a number of factors some well known others still obscure The following factors are known to be of importance (1) Electrolyte com position of the incubation medium Stadie and Zapp (1947) reported that the concentration of potassium and magnesium influenced the insulin effect smaller effects were obtained with high concentrations of these cations This is perhaps also the case for high phosphate concentrations (see below) whereas pH has little influence on the sensitivity between 6 3 and 7 6 (2) The effect of insulin on glucose uptake as well as on glycogen formation increases when the glucose concentration is raised from 0 1 to 1 per cent (3) The effect of insulin on glucose uptake is greater with rats fasted for 4 days than for 24 hours (Perlmutter and Greep 1948) (4) Young rats (80 to 100 gm) give far better results than adult rats Liébecq (1954) found that dia phragms from rats weighing about 110 gm show the greatest insulin effect expressed as percentage increase of glucose uptake (5) Injury to diaphragm tissue during excision and subsequent handling di minishes the insulin effect The latter is greater when hemidiaphragms are used than when the diaphragms are divided into four or eight parts (Willebrands *et al* 1951 Liebecq 1957) The amount of tissue per milliliter of medium is also a factor of some importance The greater the mass of tissue per milliliter the greater the decrease in glucose concentration in the medium Consequently the glucose up take is greater and can be determined with greater relative precision thus leading to increased sensitivity and accuracy The amount of tissue per milliliter of medium can be raised by decreasing the volume of fluid or by pooling hemi or quarter diaphragms of different rats The latter procedure gives the additional advantage that the indi vidual variation in sensitivity is eliminated to a certain extent (Perl mutter *et al* 1952) The sensitivity of tissue to insulin may vary from strain to strain although convincing evidence has not been published so far Wright (1957) compared two strains of rats and found sig nificant differences in base glucose uptake but not in sensitivity to insulin

Even when all of the above mentioned factors are standardized the sensitivity of the diaphragm preparation to insulin may vary from animal to animal and from day to day owing to as yet unknown factors A theoretical possibility is that the endogenous amount of epinephrine in the diaphragm influences the response of the dia phragm to insulin but this hypothesis has not as yet been verified

Although as has been outlined above rather large variations in sensitivity occur it can be stated with reasonable certainty that the smallest detectable concentration of insulin using glucose uptake of hemidiaphragms of 24 hour fasted Wistar rats of about 100 gm incu bated in bicarbonate buffer is about 0 1 milliunit of insulin per milliliter incubation medium Occasionally a significant effect at 5×10^{-6} units per milliliter has been observed (Willebrands *et al* 1950) From the point of view of sensitivity therefore the method is satisfactory

ACCURACY

The accuracy of the method is still rather poor The limits of error (confidence limits) have been calculated by Randle (1954c) and by Willebrands *et al* (1958a) to be about 3 and ⅓ This means that when a certain value *I* is found for an unknown concentration the probability is 0 95 that the true value is between $I/3$ and $3 \times I$ Reasons for this low reliability include (1) large variations in response of the diaphragms of different rats to the same dose of insulin or serum and (2) the logarithmic relation between the effect observed (stimulation of glucose uptake) and the insulin concentration to be determined (*see below*)

PROCEDURES

Procedures for the rat diaphragm method have been published by Groen *et al* (1952) Perlmutter *et al* (1952) Vallance Owen and Hurlock (1954) Randle (1954c) Willebrands and Groen (1956) Wright (1957) Randle (1956) and Willebrands *et al* (1958a)

Although the later procedures are basically the same as the initial ones small differences of technique may influence the results to a considerable extent Most if not all authors use Wistar strain rats weighing 100 to 150 gm which have been fasted for 24 hours All used bicarbonate buffer (Gey and Gey or Krebs Ringer) except Perl

mutter *et al* (1952) who used Krebs Henseleit phosphate buffer The glucose concentration in the medium varies from 150 to 300 mg per hundred milliliters of this buffer Most authors use hemidiaphragms although some use diaphragms divided into quarters or fifths Al though quarter diaphragms give an opportunity to test four different solutions with the tissue of the same rat(s) smaller sensitivity results Randle (1954c) Vallance Owen and Hurlock (1954) and Wright (1957) use a single hemidiaphragm technique (one hemidiaphragm in 1 or 2 ml of medium) while Willebrands and Groen (1956) and Perl mutter *et al* (1952) pool hemi or quarter-diaphragms The theoretical advantage of pooling is smaller variation in the preparation Disad vantages however include the greater number of rats required and the time lag during the excision of the diaphragms The latter may lead to diminished sensitivity Willebrands *et al* (1958a) are using a single hemidiaphragm technique which is described in detail below

Nearly all investigators use stimulation of glucose uptake as the indicator of insulin like activity Perlmutter *et al* (1952) determined glycogen synthesis This however requires more work Moreover glycogen synthesis amounts to only about 70 per cent of the glucose uptake Glucose uptake may be expressed per unit of wet weight per unit of dry weight or even per diaphragm provided the rats are of the same weight

Nearly all authors report the sensitivity of the method to be great the minimum detectable concentration of insulin being 10^{-4} units per milliliter of incubation medium or less although Perlmutter *et al* (1952) using a phosphate buffer found that 5×10^{-3} units was the smallest detectable concentration in their system

The relation between insulin concentration and glucose uptake is not a linear one The regression line obtained by plotting glucose uptake against the logarithm of the insulin concentration also shows curvature at both ends (Willebrands *et al* 1950) Randle (1954c 1956) and later Willebrands and Groen (1956) found that the cube root of glucose uptake versus the logarithm of the insulin concentration gives a linear regression line Vallance Owen and Hurlock (1954) and Wright (1957) use glucose uptake versus the cube root of the insulin concentration These mathematical manipulations are only approxi mations For the calculation of an unknown insulin concentration it is preferable to determine the regression line simultaneously with the unknown since there is appreciable day to day variation in the standard curve The slope of the regression line however was found by most investigators to be fairly constant (Randle 1954c Vallance

Owen and Hurlock 1954 Wright 1957 Willebrands *et al* 1958a) Therefore these authors run one standard solution together with the unknown to fix the position of the calibration line (single standard method) Occasionally two standard solutions are included in one test or two standards and two different dilutions of the serum to be tested (4 point assay) (Randle 1954c)

The values reported for the insulin content of normal human serum vary rather widely Perlmutter *et al* (1952) were unable to demonstrate the presence of insulin in serum Vallance Owen and Hurlock (1954) and also Wright (1957) found less than 0 1 milliunit per milliliter in the fasting state and Willebrands and Groen (1956) reported figures of 0 1 to 3 0 milliunits per milliliter while fasting and resting Randle s (1954c) values at first ranged from 10 to 20 milliunits per milliliter but later (1958) between 1 and 4 milliunits per milliliter 2½ hours after receiving 50 gm of glucose by mouth These differences are definitively greater than the confidence limits mentioned above They are probably not or only to a small extent influenced by such factors as previous diet or anticoagulants but are at least for the greater part caused by the concentration of serum in the incubation medium Groen *et al* (1952) Randle (1954c) and Willebrands and Groen (1956) used serum diluted four to five times with buffer whereas Perlmutter *et al* (1952) used serum diluted twice and Vallance Owen and Hurlock (1954) and Wright (1957) used undiluted serum It has since been shown by Randle (1957) and by Willebrands *et al* (1958a) that significantly lower figures for serum insulin are obtained with undiluted serum than with serum diluted five to ten times with buffer The Amsterdam group has suggested that this dilution phenomenon is caused by an insulin antagonist perhaps epinephrine (Groen *et al* 1958) the anti insulin activity of which diminishes more rapidly with dilution than the insulin effect Another possibility is that insulin circulates partially in a less active bound form which dissociates when diluted Since in the original method the values obtained by the authors tend to be constant at higher dilutions the serum dilution has been standardized at 1 10 in the method described below

RECOMMENDED PROCEDURE*

In five parallel experiments glucose utilization is estimated for the two halves of the same diaphragm one half being incubated with

* Willebrands *et al* 1958a

a known concentration of insulin and the other with the diluted serum to be tested In the calculation it is assumed that a linear relationship exists between glucose uptake and the logarithm of the insulin concentration and that the slope of the regression line is constant The standard included in the determination is used to fix the position of the regression line

Reagents

(1) Buffer solution according to Gey and Gey

Stock solution I 280 gm NaCl 14 8 gm KCl 12 0 gm $MgSO_4$ $6H_2O$ per liter

Stock solution II 90 8 gm $NaHCO_3$ 6 0 gm Na_2HPO_4 $2H_2O$ 1 2 gm KH PO_4 180 mg phenol red per liter

To obtain the solution to be used dilute 25 ml stock solution I plus 25 ml stock solution II with distilled water to about 900 ml add 2 ml of a 25 per cent weight per volume solution of $CaCl_2$ $6H_2O$ gas with 93 per cent O_2 to 7 per cent CO_2 and make up to 1 liter *

(2) Crystalline insulin 20 to 25 units per milligram

(3) Glucose anhydrous

Test

Five Wistar rats weighing 110 ± 10 gm fasted for 24 hours are killed at 5 minute intervals by a blow on the head followed by decapitation The diaphragms are excised rapidly with the least possible trauma by cutting close to the ribs The tissues are then suspended for 5 minutes in about 50 ml ice cold Gey and Gey buffer (without glucose) while gassing with 93 per cent O_2 to 7 per cent CO_2 to remove blood etc The washed diaphragms are then spread on a piece of hardened filter paper blotted lightly and trimmed The remaining thin portion is then divided into two equal parts which are transferred to Warburg flasks or 25 ml Erlenmeyer flasks already containing the standard or the unknown solution The standard flasks contain 1 0 ml of Gey and Gey buffer with 150 per cent of glucose and 0 5 milliunit of insulin per milliliter freshly prepared by dissolving a few milligrams of the crystalline insulin in as little as possible 0 001 N HCl and diluting with buffer containing 150 mg per hundred

* This solution contains 148 mEq Na^+ 5 2 mEq K^+ 4 6 mEq Ca^{++} 2 6 mEq Mg^{++} 130 mEq Cl^- 27 mEq HCO_3^- 1 2 mEq $PO_4^=$ and 2 6 mEq $SO_4^=$ per liter

milliliters of glucose to the desired insulin concentration The unknown flasks contain 1 0 ml of the buffer and serum to be tested The glucose concentration in the serum is determined in advance and calculated volumes of buffer without glucose and/or buffer containing 500 or 150 mg per hundred milliliters of glucose are added until the serum concentration is 10 per cent and the final glucose concentration 150 ± 5 mg per hundred milliliters

All flasks are put in ice water and the incubation media are equilibrated with a gas mixture of 93 per cent O_2 to 7 per cent CO_2 prior to the introduction of the diaphragms The flasks are then gassed once more for a few minutes stoppered and incubated at 37°C with shaking (120 cycles per minute) for 90 minutes. The flasks are then removed and each incubation fluid is poured into a tube and placed on ice After all the incubation media have been collected (ten in all) the glucose concentration in these media is determined as accurately as possible together with the initial glucose concentration in the buffer serum mixtures. Any method for glucose determination may be used provided its specificity is good and its accuracy is high The accuracy is of special importance because the decrease in glucose concentration amounts to only 20 to 70 mg per hundred milliliters The *differences between the initial and final glucose concentrations give* the figures for the absolute glucose uptake per hemidiaphragm (in milligrams) times 100 which are used in the calculation *

Calculations

Let G be the glucose uptake in a medium with the standard insulin solution I_s (= 0 5 milliunits per milliliter of medium)

Let G_x be the glucose uptake in a medium with the unknown insulin concentration I_x Then $(G_x - G_s)_M$ is the mean value of the difference in glucose uptake in the five tests with I and I_x Each difference $(G_x - G_s)$ stems from two flasks containing tissue of the same rat

Assuming a linear relationship between glucose uptake and the logarithm of the insulin concentration and assuming also that the slope b of this linear regression line is the same for all rats and for

* According to Liébecq (1954) the glucose uptake of hemidiaphragms with or without insulin is independent of the weight of the diaphragms thin diaphragms of small rats take up as much glucose as thick diaphragms of heavy rats Therefore the glucose uptake per gram of incubated tissue times the weight of the tissue per flask i e the glucose uptake per flask is essentially constant

all days I_x can be calculated by the equation

$$D = \log I / I_s = (G_x - G)_M / b \qquad (I_x = I \quad 10^D)$$

or

$$I_x = I_s \text{ antilog } (G - G)_M / b \qquad (1)$$
$$= 0\ 5 \text{ antilog } (G - G_s)_M / b \text{ milliunits per milliliter medium}$$

The value of b can be obtained by performing a number k of tests with two known but different concentrations of insulin I_{s1} and I_{s2} for example, $I_{s1} = 0\ 5$ and $I_{2} = 0\ 125$ milliunits per milliliter In this case $\log I_1/I_2 = 0\ 6$ and $b = (G_1 - G_2)_M/0\ 6$

These tests may be repeated over the concentration range 0 03 to 8 milliunits per milliliter at the same ratio of 4 to check linearity

The mean value of b obtained in all k^o experiments may be used in equation (1) for the calculation of I_x

The confidence limits for $D(D_1$ and $D_2)$ can be calculated* by the formula

$$D_1 \quad \text{and} \quad D_2 = \frac{(G_x - G)_M}{b} \pm \frac{t}{b} \sqrt{a}$$

in which

$$a = \frac{(k - 1)(sd\Delta G)^2 + (n - 1)(sd\Delta G)^2}{(k^o + n - 2) \quad n}$$

where

t = Student s t which at $F = k^o + n - 2$ degrees of freedom has a probability P

k = number of tests with I_1 and I_2

n = number of tests with I and I (In description above $n = 5$)

$(sd\Delta G)_s$ and $(sd\Delta G)_x$ are standard deviations† in the k tests with I_1 and I_2 in the n tests with I and I respectively

The confidence limits for I_x are therefore

$$I \quad 10^{D_1} \qquad \text{and} \qquad I \quad 10^{D_2}$$

By multiplying I and the confidence limits for I_x with 10 the value of the serum insulin concentration (in milliunits per milliliter) with confidence limits are obtained

* For an alternative method for the calculation of these confidence limits see Willebrands *et al* (1958a)

† sd = standard deviation

Remarks

(1) When high or low serum insulin values are expected a standard insulin solution of 2 or 0 1 milliunits per milliliter should be used instead of 0 5 milliunit per milliliter In these regions the linearity of the regression line should be checked At the lower and higher ends of this line the slope *b* is often smaller indicating curvature When extremely low insulin values are expected these can be tested on reality by taking *I* = 0

(2) When the presence of insulin antagonists is suspected it may be demonstrated by proceeding as above and in addition adding a known concentration of insulin to the serum

(3) Using the technique described the values found in serum of normal human beings while fasting were between 0 1 and 4 milliunits per milliliter As previously pointed out this wide variation does not necessarily indicate that the actual insulin content of the serum varies so widely The variation is mainly due to the limited accuracy of the method This may also explain why the method is often unable to differentiate between normal blood insulin values and those of patients with mild diabetes

Our efforts during the past years have therefore been directed mainly towards improving the method and elucidating the problem of what we actually determine rather than towards its application to the study of the secretion of insulin under physiological and pathological conditions It is obvious however that once the method has been brought to a higher degree of perfection it will be essential to apply it

(4) We have also tried the use of other tissues such as mesenteric adipose tissue but in our hands adipose tissue did not seem preferable to diaphragm tissue (v d Geld *et al* 1957) Recently however it has been claimed that epididymal fat may be preferable (Martin *et al* 1958) and it will be of considerable interest to compare the results obtained with the two tissues It will be of particular interest to determine if the insulin epinephrine antagonism established by Groen *et al* (1958) for the diaphragm applies equally to adipose tissue

Section 3

Insulin Bioassays in Vitro Using Isolated Rat Adipose Tissue*

Donald B Martin Yves M Dagenais and *Albert E Renold*†

Previous sections of this chapter have been concerned with the measurement of insulin like activity in plasma and serum based upon their biological activity *in vivo* as well as upon their effects upon rat diaphragm muscle *in vitro* This section will be devoted to the experience which has been gathered during the past two years while trying to adapt the insulin response of rat adipose tissue *in vitro* to the measurement of insulin like activity The rather short duration of our experience with this tissue is emphasized in order to underline the preliminary nature of the conclusions to be given The results gathered to date however seem sufficiently promising to warrant discussion at this time although a full comparative evaluation of the procedures based upon the activity of insulin upon this tissue as compared with the rat hemidiaphragm procedure has not as yet been carried out

That adipose tissue is not merely a passive storage tissue for excess fat has been fully appreciated since Schoenheimer s studies with deuterium (Schoenheimer 1942) particularly emphasized by the reports from the laboratories of Wertheimer (Wertheimer and Shapiro 1948 Shapiro and Wertheimer 1956 Shapiro 1957) Hausberger (Hausberger *et al* 1954 Milstein and Hausberger 1956) Favarger (Favarger and Gerlach 1955 Favarger and Bodur 1956) Fawcett (1948) and Engel (Engel and Scott 1950) The direct responsiveness of adipose tissue to insulin has been known for some years (Renold *et al* 1950 Krahl 1951 Haugaard and Marsh 1952) although it is only recently that the high degree of sensitivity and the quantitatively dramatic response of this tissue to insulin have been fully appreciated (Renold *et al* 1957 Martin *et al* 1958 Winegrad and Renold 1958a b Beigelman and Antoniades 1958) The lesser sensitivity and quantitatively less significant response reported by Itzhaki and Wertheimer (1957) and by v d Geld *et al* (1957) may well be related

* The studies upon which this review is based were supported by grants from the Adler Foundation Inc Rye New York the Capps and Proctor Funds of Harvard Medical School Boston Massachusetts and Eli Lilly and Company Indianapolis Indiana

† With the technical collaboration of Vilma Lauris

to differences in the handling of the tissue (chilling slicing repeated blotting *etc*) It is now appreciated not only that the effect of insulin upon adipose tissue occurs at very low insulin concentrations and is quantitatively an important one but that it also occurs within seconds of adding insulin to the tissue and that it is associated with the binding of insulin to the tissue (Haugaard and Marsh 1952) in a manner similar to that first described by Stadie (1954) for muscle The activity of adipose tissue towards insulin in terms of hydrolysis of the insulin molecule (insulinase type activity) appears to be relatively small (Piazza *et al* 1959) a fact which may have as a physiological corollary a more persistent effect of the hormone upon this tissue

To date experience has been gathered with the use of three parameters of insulin like activity in adipose tissue (1) glucose up take (i e glucose disappearance from the medium in the presence of adipose tissue) (2) net gas formation as measured in the Warburg respirometer and (3) the oxidation of glucose 1 C^{14} to $C^{14}O_2$ Of these (1) and (3) have progressed to the stage of being applied to measurements in biological fluids while (2) is being actively investi gated in collaboration with Dr Eric G Ball in the Department of Biological Chemistry Harvard Medical School Whatever the param eter used six fragments of epididymal adipose tissue may be easily obtained from any one rat allowing for the comparison of several unknowns and several standards in tissue obtained from a single animal obviating the need for comparing the response of the tissues of *different animals for a given assay*

GLUCOSE DISAPPEARANCE FROM THE MEDIUM

The technique using this parameter of insulin like activity is similar to the hemidiaphragm technique Briefly the *procedure is* as follows The epididymal fat of male rats fed ad libitum weighing between 200 and 250 gm is subdivided into three pieces for each side — a total of six pieces from each animal with any one piece weighing between 80 and 200 mg In obtaining the tissue adequate precautions are observed to minimize handling drying or cooling Each tissue fragment is placed directly into preweighed flasks con taining either 0 8 ml of Krebs bicarbonate buffer *containing gelatin*

and 200 mg per hundred milliliters of glucose or 0 8 ml of serum containing 200 mg per hundred milliliters of glucose The flasks are covered and reweighed gassed for 5 minutes with 5 per cent CO_2 and 95 per cent O_2 and incubated for between 2 and 4 hours in a Dubnoff metabolic shaking incubator at 37 6°C Flasks containing the same solutions but no tissue are run through the same procedure in each instance to act as controls At the end of the incubation period the glucose concentration is measured in all flasks and the glucose disappearance is calculated from the difference in glucose concentration between the control flasks and the tissue containing flasks Insulin like activity is then calculated as glucose utilized per unit weight of tissue (It has been adequately demonstrated that the weight of tissue so measured may be utilized *within the same animal* as reference measurement whereas the determination of total nitrogen or of dry fat free weight as reference measurements is preferable when comparisons are carried out between animals) The baseline activity of unstimulated tissue varies around 2 to 10 micromoles (0 4 to 1 8 mg) of glucose per gram of tissue and as much as a fourfold increase may be obtained in the presence of maximal effective insulin concentrations Reasonably satisfactory dose/response curves can be established between 10 and 1 000 microunits per milliliter although Beigelman (1959) has found that the sensitivity of the tissue is on occasion so great that 1 microunit may be detected and that 100 microunits per milliliter may lead to near maximal response

Using *undiluted* plasma* or serum normal fasting insulin like activity in man was found to range between 50 and 250 microunits per milliliter with this technique Beigelman and Antoniades (1958) using a similar technique but diluted plasma or serum (usually dilutions in the range of 1 10) frequently obtained higher values for fasting normal subjects up to 1000 microunits per milliliter Beigelman and Antoniades (1958) also reported that little or no insulin like activity was found in serum of patients with the growth onset type of diabetes and particularly in patients with diabetic acidosis although they appreciated the possibility of the presence of inhibitors of insulin like activity in such samples Humbel (1959) in an excellent recent evaluation of this procedure reported normal fasting values ranging from 20 to 800 microunits per milliliter

* Considerable evidence has been accumulated recently to indicate that commercial solutions of heparin may influence glucose utilization and CO_2 production by rat adipose tissue although it is not clear as yet whether heparin as such or other components of the rather crude extracts used commercially are involved

NET GAS FORMATION

In the Warburg respirometer net gas formation mainly consists of *the total production of CO_2 minus the oxygen consumed by the* tissue Under most conditions it would appear that oxygen con sumption by this tissue is rather constant and that it is but poorly

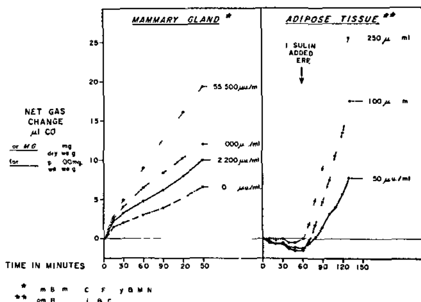


FIGURE 1 Effect of insulin added *in vitro* on net gas formation by slices of lactating rat mammary gland and by intact rat adipose tissue

responsive to the presence of insulin in the medium Therefore the major change measured as net gas formation is CO_2 production The measurement may also be considered to express changes in the respiratory quotient of the tissue The procedure is comparable to that previously used by Balmain and collaborators (1954) when ex ploring the activity of insulin on lactating rat mammary gland *in vitro* These authors were successful in establishing a satisfactory dose/response curve with insulin in mammary gland slices although the limit of sensitivity was of the order of 2200 microunits per milli liter In the case of adipose tissue the effect occurs reproducibly at concentrations in the range of 25 microunits per milliliter Compara tive curves are shown in Figure I which is modified from Ball *et al* (1959)

and 200 mg per hundred milliliters of glucose or 0 8 ml of serum containing 200 mg per hundred milliliters of glucose The flasks are covered and reweighed gassed for 5 minutes with 5 per cent CO_2 and 95 per cent O_2 and incubated for between 2 and 4 hours in a Dubnoff metabolic shaking incubator at 37 6°C Flasks containing the same solutions but no tissue are run through the same procedure in each instance to act as controls At the end of the incubation period the glucose concentration is measured in all flasks and the glucose disap pearance is calculated from the difference in glucose concentration between the control flasks and the tissue containing flasks Insulin like activity is then calculated as glucose utilized per unit weight of tissue (It has been adequately demonstrated that the weight of tissue so measured may be utilized *within the same animal* as reference measurement whereas the determination of total nitrogen or of dry fat free weight as reference measurements is preferable when com parisons are carried out between animals) The baseline activity of unstimulated tissue varies around 2 to 10 micromoles (0 4 to 1 8 mg) of glucose per gram of tissue and as much as a fourfold increase may be obtained in the presence of maximal effective insulin concen trations Reasonably satisfactory dose/response curves can be estab lished between 10 and 1 000 microunits per milliliter although Beigel man (1959) has found that the sensitivity of the tissue is on occasion so great that 1 microunit may be detected and that 100 microunits per milliliter may lead to near maximal response

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Considerable evidence has been accumulated recently to indicate that commer cial solutions of heparin may influence glucose utilization and CO_2 production by rat adipose tissue although it is not clear as yet whether heparin as such or other components of the rather crude extracts used commercially are involved

amounts of total radioactivity than would be necessary if uniformly labeled glucose were used The suggested procedure follows (Martin *et al* 1958)

Male albino rats of the Wistar strain fed ad libitum and weighing between 200 and 250 gm are killed by a blow to the head and rapid exsanguination The epididymal adipose tissue is gently taken up at its base (just distal to the epididymal vessels) cut free (single cut) and without manipulation or chilling further cut into three equal pieces Each piece weighing 80 to 200 mg is dropped as it is cut into appropriate 10 ml rubber stoppered flasks which have been preweighed and contain 2 ml of plasma or 2 ml of Krebs bicarbonate buffer containing gelatin 200 mg per hundred milliliters and insulin in appropriate dilutions The glucose concentration of both buffer and plasma samples is made up to 300 mg per hundred milliliters containing 0 2 microcuries of glucose 1 C^{14} (1 9 ml buffer or plasma plus 0 1 ml glucose 1 C^{14} solution) The rubber stoppers are provided with two ports (glass tubing with rubber fittings) a small plastic cup being suspended beneath one of these ports Alternatively serum sleeve type rubber stoppers similarly provided with a small suspended plastic cup may be used

After reweighing to obtain the tissue weight the flasks are gassed (using No 21 needles through the small rubber fittings) with 5 per cent CO to 95 per cent O_2 for 5 minutes The needles are then withdrawn (leaving the system airtight) and the flasks are incubated for 2 hours at 37 6 C in a Dubnoff metabolic shaking incubator at 72 cycles per minute After incubation using a tuberculin syringe and No 21 needle 0 1 ml of sodium hydroxide freshly diluted 1 10 from saturated sodium hydroxide with CO_2 free water is introduced through the appropriate port into the suspended cup and 0 2 ml 10 NH_2SO_4 is introduced into the medium After at least 2 hours the contents of the suspended cup are transferred quantitatively to a weighed centrifuge tube containing 1 2 per cent barium chloride and 2 7 per cent ammonium chloride and the precipitated barium carbonate is washed three times with CO_2 free water and dried and reweighed to give the total barium carbonate present in the flask (usually 15 to 25 mg) It is important to check for possible leaks which will decrease CO_2 recovery

An aliquot of each sample is then transferred to a weighed planchette for determination of its specific activity in a proportional flow counter giving counts ranging from several hundred to several thousand

As for the measurement of glucose disappearance from the medium rats weighing between 200 and 250 gm and tissue fragments weighing between 80 and 200 mg have been found optimal in this technique Again six fragments are obtained from each animal rapidly weighed (in this instance on a torsion balance) then placed in Warburg flasks without a center well containing Krebs bicarbonate buffer 200 mg of gelatin and 300 mg of glucose per hundred milliliters After equilibration the flasks are taken down and either standard insulin solutions in gelatin and glucose containing Krebs bicarbonate buffer or glucose containing sera are substituted for the control solutions Simultaneously an appropriate amount of insulin to subsequently give a final concentration of 0 1 unit per milliliter (100 000 micro units per milliliter) may be added to the side arm Incubation is then carried out for 1 to 1½ hours gas production being measured every 10 minutes The side arm is then tipped and a third incubation period is carried out to estimate the maximal insulin response of the tissue over a period of ½ to 1 hour With this technique therefore it seems possible to evaluate for each fragment of adipose tissue its baseline activity in the presence of glucose its maximal responsiveness to large doses of insulin and its response either to the unknown solution to be tested or to standard solutions of intermediate strength Thus the response to an unknown concentration of insulin like activity may be expressed in terms of per cent of maximal response a procedure which may well prove to have very significant theoretical advantages Adequate studies with biological samples have not however as yet been completed and the results obtained will have to be awaited before intelligent evaluation may proceed

FORMATION OF $C^{14}O_2$ FROM GLUCOSE 1 C^{14}

Up to the present time the largest experience accumulated with adipose tissue as a detector of insulin like activity has been gained from following this metabolic parameter which we have found to be more reliable than glucose uptake The measurement was selected empirically on the basis of studies of adipose tissue metabolism which revealed that the greatest increment over baseline in the presence of insulin could be obtained with this particular measurement (Winegrad and Renold 1958a b) probably reflecting the high degree of phosphogluconate-oxidative pathway activity in this tissue The use of glucose labeled in carbon 1 permits the use of smaller

In dogs insulin like activity was found to be consistently higher in samples obtained from the pancreatic or portal vein than in simul taneously obtained samples from the femoral vein * In normal human

TABLE IV Insulin like Activity of Human Plasma or Serum as Measured with Rat Adipose Tissue *in Vitro* Preliminary Data Obtained with Undiluted *Samples*

Metabolic state of subjects or patients	Sample assayed	Number	Insulin like activity (microunit per ml sample) Mean	Range
Normal, fasting	Plasma*	28	506	27 2500
Normal fasting	Serum	31	251	5 822
Normal + glucose i v (0 5 gm /kg /3 minutes)				
Control	Serum	6	100†	—
10 minutes	Serum	6	160†	4 333†
20 minutes	Serum	6	514†	228 - 1000†
60 minutes	Serum	6	86†	4 168†
Normal + insulin i v (0 1 unit/kg)				
Control	Plasma	3	100†	—
10 minutes	Plasma	3	456†	366 506†
30 minutes	Plasma	3	64†	37 113†
Untreated diabetes (adult onset type)	Serum	15	319	30 - 1000
Insulinoma				
(1) Preoperative	Serum		855	
Postoperative	Serum		99	
(2) Preoperative	Serum		800 >1000	
Postoperative	Serum		140	
(3) Preoperative	Plasma		320 690 85	
Postoperative	Plasma		142 115	
(4) Preoperative	Serum		>1000	
Postoperative	Serum		133	

* See footnote page 78
† Expressed as per cent of control value

subjects insulin like activity was as a rule higher after glucose ad ministration than before and 4 patients with proven insulin secreting tumors were found to exhibit abnormally high values before surgery

* These studies were carried out in collaboration with Dr E F Pfeiffer of the University of Frankfurt am Main when he was in the Department of Medicine at the Peter Bent Brigham Hospital

per minute Results are expressed as counts per minute per total CO_2 (as barium carbonate) per hundred milligrams of adipose tissue *

As a routine eighteen flasks are carried through the procedure in any single instance and the comparison of four unknowns and two stand ards is carried out in each of the three animals (six pieces of tissue from each animal) thus allowing *for routine triplicate analysis* of unknown samples in any single run The limit of sensitivity is 10 microunits per milliliter The variation in $C^{14}O_2$ production in replicate tissue samples with and without insulin is 8 to 12 per cent the maximal range (lowest to highest value of sextuplicate deter mination as per cent of the mean) 18 to 35 per cent The index of precision (Lambda) for dose response curves from 31 to 500 microunits per milliliter is found to vary between 0 20 and 0 35 for individual groups of 10 to 20 assays As the number of determinations is increas ing it is hoped that a more complete statistical evaluation will soon be available

Using this last technique a considerable body of information has been accumulated which shall now be briefly reviewed (also reviewed *in Tables III and IV*)

TABLE III Insulin like Activity of Plasma Simultaneously Drawn from Pancreatic Acid and Femoral Vein in Dogs

Experiment No	Insulin like activity (microunits per ml of plasma)	
	Femoral vein	Pancreatic vein
1	190	720
2	380	1650
3	155	300
4	240	275
5	90	810
6	275	1110
7	310	590
8	260	2190
9	30	220
10	30	340
11	360	715

* In more recent experiments it has been found more practical to transfer the total CO containing alkali from the plastic cup directly to pre weighed planchettes After slow careful drying on a hotplate these planchettes may be counted directly thus directly providing an estimate of total CO_2 radioactivity

or may not have when compared with the hemidiaphragm technique the ability to study insulin like activity of biological fluids with *two* tissues as test objects should improve our attempts to unravel the mechanisms and controlling factors underlying the secretion of insulin and its transportation and disposition by the blood

with reversion to normal values after surgery concurrent with clinical improvement Excess insulin like activity could be recovered up to between 20 and 30 minutes after the intravenous administration of exogenous insulin to normal subjects Patients with adult onset type diabetes showed normal or increased insulin like activity However the latter information is as yet to be viewed with caution since persistence of exogenously administered insulin in plasma or serum appeared to be completely unpredictable in patients previously treated with insulin and showed no correlation with evidence of insulin like activity in the patients themselves (i e blood glucose and ketone levels)

At the present time this information clearly suggests that indeed the insulin like activity measured by this technique may be due to endogenous insulin However it is fully appreciated that the measure ment is one which would be expected to reveal the algebraic sum of all insulin like and anti insulin like factors and that particularly in samples obtained from clinical pathological studies the greatest caution in interpretation is in order To date it would appear that adrenal cortical steroids *in vitro* do not significantly affect this measurement and that although effects of growth hormone and epinephrine may be demonstrated in the system they occur only at concentrations not expected to occur under physiological conditions

CONCLUSION

In conclusion therefore we would like to emphasize that adipose tissue has been shown to be at least as sensitive as skeletal muscle to small concentrations of insulin and that indeed it may be more sensitive It would seem highly likely that adipose tissue represents a major site of insulin action and that this fact should receive greater attention in our thinking about the hormone than heretofore In addition this concept may be translated into another attempt to devise a bioassay for insulin like activity using this tissue as a test object It offers advantages in terms of reproducible sensitivity from day to day convenience and the ability to compare standards and unknowns within the same tissue Whether the parameter presently measured ($C^{14}O_2$ from glucose 1 C^{14}) is the optimal one is yet to be determined Regardless of any advantage this bioassay procedure may

½ hour at room temperature and 1 to 2 hours at 4°C insulin bound to antibody has moved several inches away from the site of applica

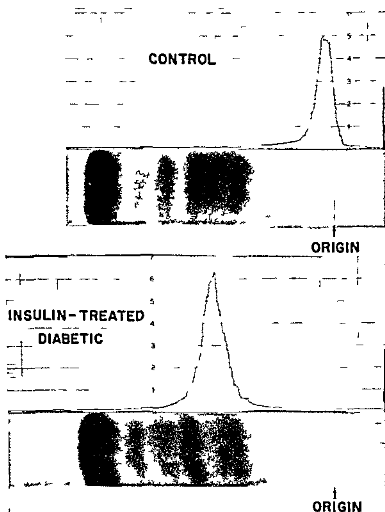


FIGURE 2 Paper radioelectrophoretograms of insulin I^{131} in plasma of control subject (top) and serum of insulin treated diabetic patient (bottom) In the control the insulin is not bound to any of the serum proteins and remains at the site of application (origin) where it has been adsorbed to the paper In the immune serum insulin is bound to antibody migrating with the front of the γ globulins Water flow chromatography alone is sufficient to separate bound from free insulin when electrophoretic resolution of the serum proteins is not of interest

tion (origin) to the paper whereas free insulin remains adsorbed to the paper at the origin The paper strips are dried and assayed for

Section 4

Immunoassay of Insulin*

Solomon A Berson and *Rosalyn S Yalow*

Few attempts have been made to apply immunological procedures to the assay of insulin probably in part owing to the general im pression that insulin is a poor antigen (Lerman 1944 Haurowitz 1950) Moreover the extremely low concentrations in which insulin exists in blood and other biological fluids might be expected to impose difficulties in its detection even if antisera were readily avail able However two methods for the immunoassay of insulin have been reported recently One of these (Berson and Yalow 1958) em ploys the reaction between I^{131} labeled insulin and the insulin binding antibodies observed to be present in the sera of virtually all insulin treated human patients (Berson *et al* 1956) (Fig 2) The other method depends on the ability of antisera to inhibit lysis of insulin sensitized erythrocytes (Arquilla and Stavitsky 1956a)

In the former reaction the extent of binding of added tracer insulin I^{131} to antibody in the presence of varying concentrations of insulin has been shown to follow the law of mass action and to be dependent on the concentrations of insulin and antibody and on equilibrium constants which differ in individual antisera (Berson and Yalow 1957 1958) For the purpose of insulin assay the quantitative relationships are most conveniently expressed in terms of the ratio of bound (B) to free (F) insulin I^{131} as a function of the total con centration of insulin (I) The ratio B/F decreases in a curvilinear fashion with increase in (I) Separation of bound and free insulin is effected by water flow paper chromatography (Berson *et al* 1956)

A series of standard insulin solutions is prepared over an appro priate concentration range and to each is added tracer insulin I^{131} in concentrations small compared to that of the unlabeled insulin Tracer insulin I^{131} is added also to unknown solutions Antiserum in appropriate dilution is then added to each solution and the mix tures are incubated at 37°C for 2 to 4 hours following which an aliquot is applied to paper strips for chromatography After about

We are greatly indebted to Doctor O H Behrens and Doctor C W Pettinga and the Eli Lilly Laboratories for crystalline beef and pork insulins to Doctor Hans Neurath for crystalline horse insulin and to Doctor F Tietze and Doctor J Field for the preparation of human insulin used in these studies

radioactivity in an automatic strip counter (Fig 3) The areas corresponding to the peaks of free insulin and bound insulin are determined and the results are plotted as B/F versus (I) (Fig 4) The insulin concentration in an unknown sample is that which corresponds to the standard solution with the same B/F ratio (see Fig 4) The

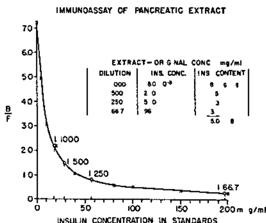


FIGURE 4 Ratio of bound insulin to free insulin (B/F) as a function of insulin concentration in known standards (λ) and B/F ratios in unknowns (0) plotted along the standard curve The insulin concentration in an unknown is determined from the horizontal axis co ordinate corresponding to the observed B/F value

results of an experiment with a crude extract of beef pancreas of unknown insulin content are shown in Figures 3 and 4 Here the extract was assayed at several different concentrations selected to yield a range of B/F values corresponding to different regions along the standard curve By this procedure the precision of the determination is increased The values obtained for three such extracts by the immunoassay method were in reasonable agreement with estimates derived from mouse convulsion tests (Table V)

The disappearance of exogenously administered insulin from the circulation of rabbits has been evaluated by a similar technique One unit of unlabeled crystalline beef insulin was administered intravenously to each of two animals and at intervals thereafter plasma was assayed for its beef insulin content in the manner described above The curves of disappearance showed good agreement with the disappearance of intravenously administered insulin I^{131} in a group of nine other rabbits (Fig 5)

In both of the experiments described I^{131} labeled beef insulin was

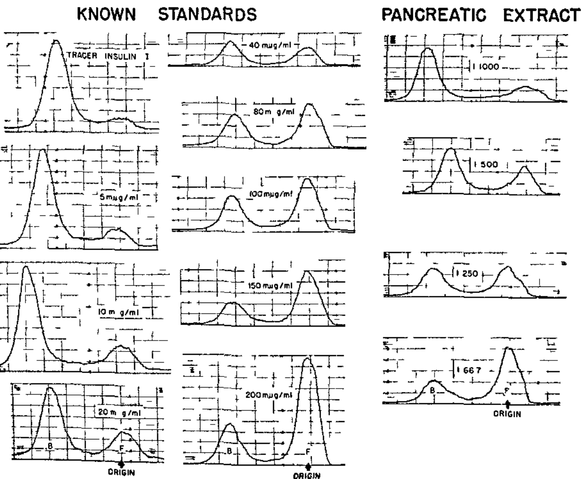


FIGURE 3 Paper radiochromatograms of insulin I131 in known standard solutions of insulin and in solutions of a crude extract of beef pancreas Free insulin is adsorbed at the origin whereas insulin bound to antibody has moved with the serum proteins on chromatographic development

the standard insulin solutions are prepared from insulin of the same species Standard curves for crystalline pork beef and horse insulin and crude human insulin employing pork insulin I^{131} and horse insulin I^{131} as tracers are shown in Figure 6 Pure insulin is not required for the standard if the insulin content of the standard preparation is known However a pure crystalline insulin should be employed for preparation of the tracer insulin I^{131} to avoid iodination of noninsulin impurities

The sensitivity for insulin detection is maximal at the lowest insulin concentrations since in this region the slope of B/F versus (I) is greatest It is a frequent finding that antisera from insulin treated subjects will show at appropriate dilutions a measurable decrease in B/F on addition of 1 millimicrogram (27 microunits) of beef insulin per milliliter above the tracer insulin I^{131} concentration Since only 50 microliters of solution containing 50 micromicrograms of insulin I^{131} of high specific activity (>50 millicuries per milligram) is more than adequate for counting the paper chromatograms such antisera can be used for the assay of as little as 50 to 100 micromicrograms (1½ to 3 microunits) of insulin However since human insulin reacts much less strongly with human antisera (as might have been anticipated) far more human insulin than beef pork or horse insulin is required to produce the same competitive decrease in binding of any of the I^{131} labeled animal insulins (Berson and Yalow 1959) The assay of human insulin in the low concentrations which generally obtain *in vivo* is therefore not feasible with these techniques With sensitive antisera a tenfold concentration of human insulin in plasma might bring the insulin concentrations into the detectable range However at present it seems more promising to investigate antisera from animals immunized with various crystalline species specific insulins in the hope that human insulin might react with such antisera more strongly than with human antisera

Another immunological insulin assay has been reported by Arquilla and Stavitsky (1956a) Employing antisera from rabbits immunized with alum precipitated beef insulin these workers found that the amount of hemolysis of insulin sensitized erythrocytes* varied inversely as the logarithm of the amount of insulin preincubated with the antisera Thus the assay depends on the ability of antisera to bind insulin and consequently to inhibit lysis of insulin sensitized cells It was possible to detect by these methods 0 1 μg of beef insulin

* Insulin is coupled to the erythrocytes with bis diazotized benzidine

TABLE V Assay of Insulin Content in Extracts of Beef Pancreas*

Extract	Mg insulin per 100 mg powdered extract	
	Immunoassay	Mouse convulsion assay
A	1 27 ± 14	1
B	97 ± 05	1
C	4 27 ± 21	5

*The extracts were provided by Dr Reuben Schucher who also performed the mouse convulsion assays

employed for the assay of beef insulin It is important to note that insulin content as measured by immunoassay is not necessarily related to hormonal activity *per se* but is specific for the insulin of a par

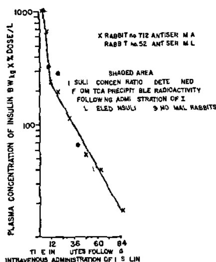


FIGURE 5 Immunologically determined plasma concentration of crystalline beef insulin (X 0) as a function of time following intravenous administration to each of 2 rabbits compared with plasma concentrations of trichloracetic acid precipitable I131 (shaded area) following intravenous administration of insulin I131 in 9 other rabbits The flattening out of the latter curves is due to the presence of damaged fractions which bind nonspecifically to plasma proteins (Berson *et al* 1956) Reproduced from Berson and Yalow (1958) *Advances Biol and Med Phys* VI p 349 Academic Press New York

ticular species Different species specific insulins react differently with human anti (beef pork) insulin sera produced in response to therapy with commercial preparations However any I131 labeled insulin may be employed for the assay of any species specific insulin provided

in 0 1 ml of solution (1 μg or 27 milliunits per milliliter) Although these amounts are about 500 to 1000 times as great as can be detected with the insulin I^{131} binding technique it is probable that the hemolytic method can be made more sensitive Human insulin appeared to react with rabbit anti beef insulin serum less well than did any of the other insulins in the hemolytic system but this was thought possibly to be an artifactual result of the cresol present in the human insulin preparation (Arquilla and Stavitsky 1956b)

In summary immunological methods are presently available for the precise assay of several different species of animal insulins in micro microgram or low millimicrogram quantities However the assay of endogenous insulin in man by these techniques must await further improvements in sensitivity

Addendum

Since the above was written immunoassay of endogenous insulin in human plasma has become feasible with antisera from guinea pigs immunized with protamine zinc beef insulin In these antisera human insulin competes almost as well as beef insulin against the

TABLE VI Standardization for Human Insulin Assay

H m ins lin (mi ro nits/ml)	B/F (b f I li I^{131})	Subj ct W		
		Gl o t l t t 100 gm gl co p	B/F t 1 4 dil u f plasm	Ins li nc t n in und l ted plasm (m nits/ml)
0	2 20	Fa ti g	1 50	100
5 6	1 88	1/2 h	1 29	142
11 2	1 85	1 h	87	300
16 8	1 69	2 hou	87	300
28	1 42			
56	1 01			
112	71			
224	53			
280	49			

binding of beef insulin I^{131} Thus the concentration of human insulin in the plasma of normal fasting subjects is more than adequate to reduce significantly the B/F ratio obtained with trace amounts of beef insulin I^{131} alone Table VI gives B/F ratios for a standard human insulin curve and for plasma samples taken during a glucose tolerance *test in subject W*

Several technical details should be observed carefully in the assay of very low insulin concentrations in plasma The antiserum should

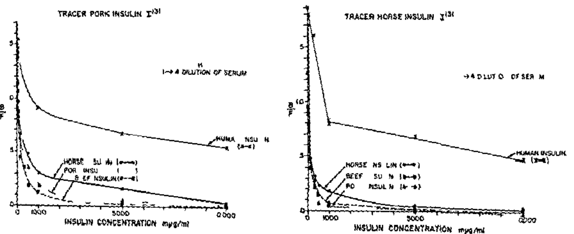


FIGURE 6 *B/F* ratios for pork insulin I131 (*left*) and horse insulin I131 (*right*) in the presence of different concentrations of beef pork horse and human insulin (Figure on top reproduced from Berson and Yalow *Diabetes* R H Williams (ed) Paul B Hoeber and Co in press)

Anti Insulin Factors*

Maurice E Krahl

In performing its physiological function insulin first becomes attached to muscle or other responsive tissue and there initiates structural changes which favor glucose uptake and synthesis of glycogen protein fat and other products (Stadie 1954 Levine and Goldstein 1955 Krahl 1957) Anti insulin factors may act by combining with the insulin and thus withhold it from the point of attachment by combining with the tissue and preventing the insulin from becoming attached or by preventing the attached insulin from initiating the structural changes which are concerned with its function These concepts are useful in accounting for differences in the results of various tests for anti insulin activity

TESTS FOR ANTI INSULIN FACTORS

In test (a) where a mixture of the factor and insulin is injected into an animal the insulin is partitioned between a relatively small quantity of anti insulin factor and a relatively large quantity of reactive tissue with a high avidity for insulin the test is therefore relatively insensitive and detects only strong anti insulin activity In test (b) where rat diaphragm muscle is exposed continuously to serum containing insulin just sufficient for a physiological stimulus the insulin is partitioned between a relatively large amount of anti insulin factor and a relatively small amount of reactive tissue the test is therefore relatively sensitive and reveals anti insulin activity where grosser methods do not Test (c) is a variant of (b) In both (b) and (c) the anti insulin factor has a chance not only to bind and withhold insulin but also to interfere with its action at the cell surface In tests (d–f) employing insulin I^{131} the insulin is partitioned between the anti insulin factor and the water phase surrounding it no tissue with high avidity for insulin is involved thus the tests are more sensitive than (a) they do not however detect some factors revealed by (b) because the possibility of interfering with insulin

Experimental work of the author has been aided by grants from the Life Insurance Medical Research Fund from Eli Lilly and Company and from the Dr Wallace C and Clara A Abbott Memorial Fund of the University of Chicago

be diluted sufficiently for the *B/F* ratio to be between 1 and 3 with tracer beef insulin I^{131} alone in the absence of human insulin Anti sera should be selected so that the *B/F* ratio falls *at least* 50 per cent in the presence of less than 100 microunits of human insulin per milli liter The concentration of tracer beef insulin I^{131} should be kept below 10 to 15 microunits per milliliter for this purpose specific activi ties of at least 40 to 50 millicuries I^{131} per milligram of insulin are required Finally insulin I^{131} may be damaged during incubation with plasma or serum with the formation of labeled components which bind indiscriminately to serum proteins Therefore control mixtures should be made without antiserum to permit correction for non specific binding of damaged fragments Since the antiserum will generally be used at a dilution of 1 200 to 1 5 000 or greater depend ing on its potency damage by antiserum is not a problem

(k) Inhibition of enzymes without or with reversal of inhibition by insulin (Christenson *et al* 1949 Weil Malherbe 1950 Krahl and Bornstein 1954 Bornstein 1956 Il in and Titova 1956)

PROPERTIES OF ANTI INSULIN FACTORS

Factors Related to Previous Insulin Administration

A substance combining with insulin I[131] appeared in the blood of nearly all subjects treated for a sufficient time with commercial insulin (Berson *et al* 1956 Berson and Yalow 1957a Skom and Talmage 1958) The anti insulin activity by a number of tests was accentuated when the subject developed resistance or allergy to beef or hog insulin* such activity decreased when the insulin treatment was discontinued or when resistance fell after ACTH administration (Marsh and Haugaard 1952 Colwell and Weiger 1956)

This anti insulin factor moved electrophoretically as a γ-globulin or in the region between β globulins and γ-globulins (DeFilippis and Iannaccone 1952 Kaye *et al* 1955 Sehon *et al* 1955 Colwell and Weiger 1956 Koenig *et al* 1956 Berson and Yalow 1957a Burrows *et al* 1957) It was relatively stable to icebox storage could be lyophilized and was not destroyed by heating to 56°C for 24 hours

Since this factor has some properties of an antibody its specificity toward insulin of various species has been studied (Moloney and Coval 1955 Arquilla and Stavitsky 1956b Burrows *et al* 1957 Yalow and Berson 1957) The binding of crystalline beef insulin I[131] by antibody from a patient treated with crystalline beef insulin was not reduced by human insulin (Burrows *et al* 1957) the specificity was therefore in this instance primarily to the foreign protein rather than to the insulin On the other hand antibodies which were developed in animals to exogenous insulin of various species showed strong cross reactions the cross reaction to human insulin was relatively weak (Moloney and Coval 1955 Arquilla and Stavitsky 1956b) Antibodies with reactivity to human insulin may be useful in assay of insulin in human plasma (Arquilla and Stavitsky 1956a Yalow and Berson 1958)

* Banting *et al* 1938 Glen and Eaton 1938 Marble *et al* 1940 Goldner and Ricketts 1942 Wasserman and Mirsky 1942 Lerman 1944 Lovell 1944 1947 DeFilippis and Iannaccone 1952 Marsh and Haugaard 1952 Kaye *et al* 1955 Loveless and Cann 1955 Moloney and Coval 1955 Sehon *et al* 1955 Arquilla and Stavitsky 1956b Colwell and Weiger 1956 Koenig *et al* 1956 Steigerwald and Spielman 1956 Burrows *et al* 1957 Skom and Talmage 1958

action is not present The question whether the partition of insulin I^{131} between substances which react with it is identical with that for insulin without iodine has been raised and cannot be settled until highly radioactive insulin labeled in a normal constituent atom is available

Discussion of these different tests is limited to substances which have been evaluated by *in vitro* methods Because of space limitations it has not been possible to refer to every publication

(a) Interference with the hypoglycemic effect of known doses of insulin in mice rats rabbits or guinea pigs (Banting *et al* 1938 Glen and Eaton 1938 Lowell 1944 1947 DeFilippis and Iannaccone 1952 Kaye *et al* 1955 Moloney and Coval 1955 Sehon *et al* 1955 Colwell and Weiger 1956 Arquilla and Stavitsky 1956b)

(b) Negation of stimulating effect of insulin on glucose uptake by rat diaphragm muscle (Bornstein and Park 1953 Bornstein 1953 Vallance Owen and Hurlock 1954 Randle 1954c 1956 1957 Vallance Owen *et al* 1955 Baird and Bornstein 1957 Hendley *et al* 1957 Krahl 1957 Krahl *et al* 1959 Vallance Owen 1957 Vallance Owen and Lukens 1957 Willebrands *et al* 1957 1958a)

(c) Negation of stimulating effect of insulin on glycogen synthesis by rat diaphragm muscle (Marsh and Haugaard 1952 Field and Stetten 1956 Field *et al* 1957)

(d) Precipitation of I^{131} labeled insulin with β globulin and γ globulin fraction (Berson *et al* 1956 Berson and Yalow 1957a)

(e) Binding of I^{131} or S^{35} labeled insulin during electrophoresis or ultracentrifugation (Berson *et al* 1956 Koenig *et al* 1956 Berson and Yalow 1957a Bregman and Krahl 1957 Burrows *et al* 1957)

(f) Precipitation of human globulin insulin complexes with antiserum to human γ globulin (Skom and Talmage 1958)

(g) Agglutination of insulin coated red cells or particles reactions of the complement fixation type and precipitation tests (Goldner and Ricketts 1942 Wasserman and Mirsky 1942 Lerman 1944 Arquilla and Stavitsky 1956a Steigerwald and Spielman 1956)

(h) Interference with response of sensitized skin to insulin (Loveless and Cann 1955)

(i) Delay in destruction of I^{131} labeled insulin (Yalow and Berson 1957)

(j) Production of hyperglycemia by antibodies to insulin (Moloney and Coval 1955)

(k) Inhibition of enzymes without or with reversal of inhibition by insulin (Christenson *et al* 1949 Weil Malherbe 1950 Krahl and Bornstein 1954 *Bornstein 1956 Il in and Titova 1956*)

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Factors Related to Diabetic Acidosis

Anti insulin activity by test (c) was found in serum of 5 of 7 diabetic patients who were in severe acidosis with insulin resistance it disappeared when the acidosis was corrected The factor was not correlated with previous insulin therapy (Field and Stetten 1956 Field *et al* 1957) The α_1 globulin fraction was most active β globulins had some activity the lipoproteins obtained by flotation were not inhibitory after 1 day of centrifuging and dialysis The factor was stable to freezing and to heating at 60°C for 15 minutes but not at 100°C for 4 minutes it was destroyed by chymotrypsin but not by trypsin it did not interfere with the attachment of insulin I^{131} to diaphragm muscle it had no glucagon or insulinase activity

Anti insulin activity by test (b) was found in the residue after extraction of insulin from the serum of 2 acidotic patients with an ethanol toluene butanol mixture (Baird and Bornstein 1957) it was stable to extended storage

Factors Not Related to Insulin Administration or Acidosis

The plasma (undiluted) from diabetic patients of the juvenile type was found by test (b) to interfere with 0 0005 to 0 0010 unit of exogenous insulin per milliliter The factor was originally found electrophoretically as α_2 or β globulin (Vallance Owen and Hurlock 1954 Vallance Owen *et al* 1955) but later was reported to be in the albumin fraction and separable from it (Vallance Owen *et al* 1958) It was stable to extended icebox storage and to repeated freezing at −55°C and thawing at 37°C

Even normal human serum appears to mask the effects of endogenous insulin The insulin *activity* increased from about 0 0001 unit up to as much as 0 003 unit per milliliter or more as the serum was diluted (Randle 1954c 1956 1957 Willebrands *et al* 1957 1958a) Thus the partition of endogenous insulin between some component of the serum and the diaphragm muscle apparently changed in favor of the latter as the serum was diluted The factor responsible was unstable to storage at 5 C and destroyed by repeated freezing and thawing (Randle 1957) In test (b) 0 05 μg of epinephrine nullified the effect of 0 0005 unit of insulin per milliliter (Willebrands *et al* 1957)

Diabetic or Normal Animals

An anti insulin factor has been prepared from diabetic or normal rat serum and from rabbit plasma (Christenson *et al* 1949 Bornstein 1953 1956 Krahl and Bornstein 1954 Ilin and Titova 1956 Bregman and Krahl 1957 Hendley *et al* 1957) It appeared in the β globulin fraction was unstable to repeated freezing at −55 C and thawing at 60°C the fraction from 1 ml of serum nullified the action of 0 0005 to 0 001 unit of insulin per milliliter in test (b) S^{35} insulin (rabbit) when added to rat serum migrated with the β globulins (Bregman and Krahl 1957) insulin is also concentrated in the β globulin fraction of normal human plasma (Beigelman *et al* 1956a)

Anti insulin activity was found in plasma of depancreatized cats the factor was associated with the globulin fraction and prevented the action of some 0 008 units of insulin per milliliter in test (b) it was stable to repeated freezing at −55°C and thawing at 37 C (Vallance Owen and Lukens 1957) Like the anti insulin factor in *rat serum it disappeared when the animal was hypophysectomized* and for reappearance required injection of *both* pituitary growth hormone and an adrenal cortical factor such as cortisone (Bornstein and Park 1953 Vallance Owen and Lukens 1957)

There is evidence that the response of individual tissues to insulin varies *with the hormonal balance of the animal (Krahl 1951 1957* Levine and Goldstein 1955) The relation of the humoral factors discussed above to these changes is unknown

GENERAL SUMMARY

(1) The action of insulin can be influenced by several components of serum *On the basis of the present fragmentary data the situation* appears to be as follows

In the serum of normal men and animals (and some diabetics) insulin is found in and carried during electrophoresis by *the* β globulins Such β globulins can also nullify the action of insulin on muscle *perhaps* 0 001 *unit per milliliter of exogenous purified* insulin and three to twenty times as much endogenous serum insulin The stability and properties of these β globulins vary with the species and with their lipid content

In the serum of men or animals given heterologous insulin over a

sufficient period of time γ globulins (or β globulins and γ globulins) of an antibody type appear These can bind insulin during electrophoresis or ultracentrifugation and also interfere with the action of insulin on muscle When severe resistance to insulin develops these antibodies are said to combine with as much as 0 5 unit of insulin per milliliter of serum Antibodies to crystalline beef or hog insulin have relatively limited cross reactivity toward human insulin

In the serum of some human diabetics in severe acidosis still a third anti insulin factor can be detected It is associated with the α_1 globulin fraction is active against human insulin and is related to the diabetic acidosis *per se* it disappears when the subjects are returned to normal insulin control

(2) These anti insulin factors are of interest for a number of reasons They may be concerned in clinical resistance to insulin especially those of the α-globulin and γ globulin types they may interfere with the assay of insulin in serum or plasma and they may serve as prototypes for the substances with which insulin reacts to produce its characteristic physiological action upon muscle adipose tissue and other cells

Section 6

The State of Insulin in Human Plasma*

Harry N Antoniades, Paul M Beigelman and *Albert E Renold*

Complete structural information for insulin prepared from pancreas has been reported by Sanger and co-workers (Sanger 1959) Their ingenious studies have shown that bovine insulin is composed of two chains an acidic chain (A) containing 21 amino acids and a basic chain (B) containing 30 amino acids These two chains are joined together by the disulfide bridges of cystine residues (Fig 7) Differences

NH2 NH2
Ph Val.Asp Glu Hi Le Cy Gly.Se His Le Val Gl .Al Le Ty Leu Val Cy Gly Glu.Arg Gly Phe Ph Ty Thr.P Ly .Ala
(B)
S S
NH2 S NH2 S NH2
Gly Ileu V l Gl Gl Cy Cy.Al Se Val Cy.S Le Ty Gl Le Glu A p Tyr Cy.Asp
(A) S S

FIGURE 7 Structure of bovine insulin (From Sanger 1959)

in the structure of insulin from various species were found only in the three amino acids contained within the disulfide ring of the acidic chain (A)

Although the chemical structure of crystalline insulin is known little is known of the state of insulin in the untreated pancreas and also of the state in which this hormone circulates in blood Chemical methods employed for the extraction of insulin from pancreatic tissue in the effort to concentrate the active component may alter and denature the insulin complexes which occur in this tissue and which may be of important physiological significance Evidence that insulin in human and bovine pancreas is combined with other substances probably basic protein(s) has been offered by Lindner (1938) and Antoniades *et al* (1959)

The state in which insulin circulates in the blood stream may also be important for the function of this hormone It is difficult to study the physicochemical state of insulin in blood by direct isolation of the insulin and insulin complexes because of the low concentration of insulin in plasma (Bornstein 1950 *Groen et al* 1952 Randle

* Part of this work was supported by grants from the National Foundation and Eli Lilly and Company

1954c Vallance Owen and Hurlock 1954 Willebrands and Groen 1956 Beigelman and Antoniades 1958 Martin *et al*, 1958) On the basis of a molecular weight of 6000 for insulin assuming a normal insulin level of 1 milliunit per milliliter the concentration of this hormone in serum or plasma is of the order of 10^{-8} molar a concentration so small that it represents a real obstacle in the study of insulin and insulin complexes in plasma However investigators have attempted to study the state of insulin in blood by various experimental means A brief review of these findings is presented in this section

INSULIN LIKE ACTIVITY OF HUMAN PLASMA FRACTIONS

Fractions Separated with the Cold Ethanol and Zinc Fractionation Techniques

The distribution of plasma insulin like activity among plasma protein fractions was reported by Goetz *et al* (1954) and Beigelman *et al* (1956a) Pooled normal human plasma was fractionated by equilibrating the plasma with zinc ions (Surgenor *et al* 1959) and with the cold-ethanol methods of fractionation (Cohn *et al* 1946 Oncley *et al* 1949) The fractions were tested for insulin like activity by the blood sugar response of hypophysectomized alloxanized mice and rats (Goetz *et al* 1954 Beigelman *et al* 1956b) The plasma globulin precipitate (PGP) fraction prepared by equilibrating the plasma with zinc possessed insulin like activity This fraction contains primarily the β lipoproteins the γ globulins and fibrinogen The γ globulin fraction separated from this precipitate was inactive as well as the supernatant fraction (SPPS) which contains the bulk of the albumin and the α globulins and β globulins

These studies were extended by Beigelman *et al* (1956a) to plasma protein fractions of normal human plasma prepared with the cold ethanol methods of fractionation Although whole human plasma was inactive the plasma Fraction II + III tested at a threefold to sixfold concentration over the original plasma possessed insulin like activity Fractions IV 1 V and II + IIIw were devoid of any significant activity The insulin like activity exhibited by Fractions IV-4 and III 0 was equivocal It is interesting to note that the composition of Fraction II + III (β lipoproteins γ globulins) and the plasma globulin precipitate (PGP) (β lipoproteins γ globulins fibrinogen) which possessed insulin like activity is similar They both contain primarily proteins of relatively low solubility The presence of insulin

like activity in these fractions was interpreted as suggesting that plasma insulin may precipitate not as free insulin but in a complex form with other proteins the insulin complex bearing a solubility similar to the proteins present in Fractions II + III and PGP

Fractions Separated by Paper and Column Electrophoresis

Normal Plasma or Serum The presence of insulin like activity in the β globulin and γ globulin fractions has also been reported by investigators who employed paper and column electrophoresis for the separation of plasma and serum fractions Beigelman (1958) reported that the insulin like activity of serum protein fractions pre pared with the use of continuous flow paper electrophoresis was associated with the β globulin fraction Two fractions prepared by zone electrophoresis on a column of treated cellulose exerted an insulin like activity on the uptake of glucose by isolated rat diaphragm a fraction in the albumin/α_1 globulin zone and a fraction in the β globulin and γ globulin zone (Taylor 1958 Randle and Taylor 1958a b) Control experiments with ox insulin (native or I^{131} labeled) either alone or in the presence of normal human serum indicated that ox insulin migrates with a mobility corresponding to the albumin/α_1 globulin zone These findings suggested that the serum insulin like activity with the electrophoretic mobility of the albu min/α_1 globulin which is similar to the control ox insulin represents the free circulating insulin The insulin like activity of the β globu lin and γ-globulin zone suggested a combination of this portion of insulin with other serum proteins which resulted in the alteration of the electrophoretic mobility insulin in serum (Randle and Taylor 1958a b)

Similar studies carried out by Bolinger *et al* (1958a) with the use of starch column electrophoresis also showed that endogenous insulin migrates with the β globulins and to a lesser extent with the γ globulins Crystalline insulin without protein carrier migrates as an α_1 globulin However Bolinger *et al* (1958a) reported that crystal line insulin in normal serum migrates with the β globulins and γ-globulins This observation differs from that reported by Randle and Taylor (1958a b) that ox insulin in normal serum migrates in the albumin/α_1 globulin zone It is not clear to what extent these differences are due to different insulin preparations and to the dif ferences in the electrophoretic methods employed in these studies

In studies employing electrophoretic techniques for the demonstra

tion of insulin binding by serum proteins one should take into consideration the effect of at least one variable pH Electrophoretic studies at high pH values may cause partial dissociation of the insulin complexes in plasma or serum

Diabetic Plasma or Serum Berson *et al* (1956) suggested that in the plasma of insulin treated subjects insulin I^{131} is bound by a globulin which satisfies the criteria for antibody Insulin I^{131} in normal plasma or in plasma of diabetics under insulin therapy for a short period of time is in the free state Experimental data obtained with paper and starch block electrophoresis and ultra centrifuge studies showed a difference between the migration and sedimentation of the insulin I^{131} in plasma of insulin treated diabetics and that of normal plasma In plasma from insulin treated diabetics insulin I^{131} added *in vitro* or injected intravenously migrated with the γ globulins also the radioactivity of the plasma subjected to ultracentrifugation sedimented with the γ globulins The radioactivity in normal plasma migrated as an α_1 globulin and sedimented more slowly than albumin Kinetics of the reaction between beef insulin I^{131} and insulin binding antibody showed that insulin is univalent in the reaction The antibody has two distinct binding sites (Berson and Yalow 1957b) The equilibrium constants are of the order of 10^8 and 10^9 liters per mole respectively The maximal capacity for insulin binding per liter of plasma in antisera from non resistant subjects did not exceed 10 units In subjects with insulin resistance the capacity for insulin binding ranged from 80 to 400 units per liter of serum (Berson and Yalow 1957a) The latter suggested that the insulin antibody complex may serve as a source of depot insulin which is released in significant quantities over a prolonged period of time

Insulin binding by sera from insulin treated diabetics has been also demonstrated by means of the differential adsorption of insulin I^{131} by an anionic resin (Mitchell *et al* 1959) Although insulin responsive diabetics exhibited no correlation between the degree of insulin binding and insulin requirement the insulin resistant diabetics displayed tenfold to fiftyfold increases in serum insulin binding (Mitchell and O Rourke 1959)

ADSORPTION OF PLASMA INSULIN LIKE ACTIVITY ON CATIONIC EXCHANGE RESINS

Although insulin like activity was demonstrated in plasma Fractions II + III and PGP prepared from acid citrate dextrose (ACD) collected normal plasma identical Fractions II + III PGP and all other fractions prepared from plasma collected with the use of cationic exchange resin (see Chapter I) were devoid of insulin like activity This observation led to investigations of possible adsorption of the plasma insulin like activity by the cationic exchange resin during blood collection Pooled Dowex 50 cationic exchange resin following collection of normal human plasma was washed with distilled water to remove the contaminating red blood cells Elution of the insulin like activity was attempted by a variety of methods (Antoniades *et al* 1958a) Elution with 0 1 molar citrate pH 3 0 yielded a concentrate containing approximately 40 per cent of the plasma insulin like activity Elution with 0 2 molar citric acid (Antoniades 1958) and 0 1 normal sulfuric acid (Antoniades *et al* 1958b) resulted in an increased recovery of insulin from the resin

To examine whether or not the plasma insulin like activity extracted from the resin is due to plasma insulin and not to non specific factors a number of resin eluates were assayed in various laboratories by the following methods Blood sugar response in hypophysectomized alloxanized rats (Antoniades *et al* 1958a) adipose tissue assay (Dr D B Martin Dr Y M Dagenais and Dr A E Renold Baker Research Laboratory Harvard Medical School Boston) and diaphragm tissue assay (Dr H Narahara and Dr K Gundersen University of Washington Seattle Washington Dr P J Randle Cambridge University Cambridge England) The results although mostly qualitative since only a few animals were used for most assays indicated the presence of insulin activity in the resin eluates The amount of insulin in the different pools varied from 1 to 15 milliunits per milliliter of eluate These data represented a 30 to 120 per cent recovery of insulin activity as compared with the original plasma The high degree of purification of the resin eluate diminishes the possibility that the insulin activity present in the eluate is the result of nonspecific factors

Crystalline insulin dissolved in 0 15 molar sodium chloride solution or 5 per cent human plasma albumin was treated with cationic ex

change resin at pH 7 2 ± 0 4 Contrary to the results obtained with plasma insulin the crystalline insulin in 0 15 molar sodium chloride or in the albumin solution retained full biological activity after resin treatment (Antoniades *et al* 1958a) Since at physiological pH the insulin is negatively charged adsorption on cationic exchange

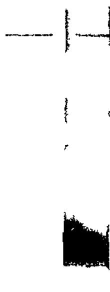


FIGURE 8 Paper electrophoresis of plasma insulin concentrate prepared with the use of cationic exchange resin (The insulin activity was associated with the A/α_1 globulin fraction The protein concentration of the resin eluate is more than 1 5 million times that of the original plasma) Left insulin eluate right plasma control (From Antoniades 1958)

resin suggests that insulin in blood circulates in a complex form with other protein(s) of higher isoelectric point probably basic protein(s) the plasma insulin basic protein complex being adsorbed on the cationic exchange resin Insulin in turn is freed from the complex by the dilute acids employed for the elution of the insulin activity from the resin A portion of plasma insulin may be in the free state suggesting an equilibrium between bound and unbound insulin in blood

Paper electrophoresis of resin eluates following blood collection was carried out at 2 C on a 4 by 31 cm paper strip (S and S 470) at pH 7 6 (phosphate buffer) and pH 8 6 (Veronal buffer) (Antoniades

1958) A control paper strip carrying plasma was developed at the end of each electrophoretic run (Fig 8) The paper strip carrying the insulin concentrate was cut in five sections the albumin the α_1 globulin the α_2 globulin the β globulin and the γ globulin section The proteins from the paper strips were extracted with bicarbonate buffer employed in the assay of insulin like activity with the adipose tissue assay and were tested for insulin like activity with the rat adipose tissue assay (Martin *et al* 1958) The insulin activity was located in the α_1 globulin section Some of the activity was present in the albumin section probably due to contamination with α_1 globulins The insulin like activity in the α_1 globulin/albumin zone probably represents the free insulin liberated from the complex by the acids employed for the elution of the insulin from the resin

THE STATE OF INSULIN IN PANCREAS

The presence of an insulin complex in pancreatic tissue as in blood has been suggested by Antoniades *et al* (1959) The suggestion was based on the observation that a considerable amount of insulin in pancreatic tissue is readily adsorbed on cationic exchange resin at pH 7 2 when pancreas homogenates in 0 15 molar sodium chloride and cationic exchange resin are mixed The insulin in turn is extracted by elution from the resin with dilute acids followed by elution with alkali (Antoniades *et al* 1959) A smaller quantity of the insulin in pancreatic tissue which escaped adsorption on the resin probably represents the free unbound insulin (Antoniades *et al* 1959) Lindner (1938) has also described that insulin extracted from fresh bovine pancreas under special conditions is poorly soluble at physiological pH and is slowly adsorbed from subcutaneous injection sites Lindner suggested that this insulin preparation termed Nativinsulin is combined with a basic protein probably a histone

POSSIBLE MECHANISM OF SECRETION AND TRANSPORT OF INSULIN

The observations on the state of insulin in blood and pancreas described in this section can be summarized as follows

(1) Insulin in the pancreas is probably bound to a large extent by basic proteins This pancreatic basic protein insulin complex is insoluble in 0 15 molar sodium chloride solution at physiological pH (Lindner 1938 Antoniades *et al* 1959)

(2) A smaller portion of insulin in the pancreas is probably in the free form representing the amount of insulin which escaped adsorption on cationic exchange resin at pH 7 2 (Antoniades *et al* 1959)

(3) Insulin in the blood stream is to a large extent bound by the plasma basic protein(s) This complex is adsorbed on cationic exchange resin at physiological pH (Antoniades 1958 Antoniades *et al* 1958a)

(4) A portion of insulin in blood which escapes adsorption on *cationic exchange resin at pH 7 2* is *probably* in the free form (Antoniades *et al* 1958b)

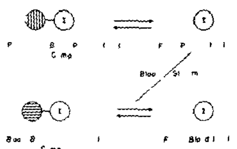


FIGURE 9 A scheme on the possible mechanism of secretion and transport of insulin

Figure 9 on the possible mechanism of secretion and transport of insulin is based on the suggestions summarized above One may speculate about the possible physiological importance of a system both in the plasma and in the pancreas providing for the likely existence of equilibria between free and bound insulin It remains to be seen whether enzyme intervention is necessary for the dissociation of insulin from its complexes in pancreas and in blood

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CHAPTER IV

Glucagon in Plasma

Maynard H Makman, Richard S Makman
and Earl W Sutherland

EXTRACTION AND PURIFICATION OF GLUCAGON

CHEMICAL AND BIOLOGICAL PROPERTIES

BIOASSAY

IDENTIFICATION AND MEASUREMENT OF GLUCAGON IN PLASMA

CONCLUSION

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While much is known about the chemical structure biological effects and mechanism of action of glucagon relatively little is known of its physiological role and possible pathological significance A hormonal role for glucagon has been suggested in the interpretation of phenomena such as the lowered insulin requirement of diabetic animals (Marks and Young 1939 Candela 1945 1952 Thorogood and Zimmermann 1945 Holt *et al* 1956) and possibly of diabetic men (Dixon *et al* 1946 Ricketts *et al* 1946) following pancreatectomy hypoglycemia occurring in normal ducks following pancreatectomy (Mialhe 1955) the hereditary obese hyperglycemic syndrome in mice (Shull and Mayer 1956) and certain hypoglycemic conditions in man (McQuarrie *et al* 1950 Froehlich *et al* 1951 Bierich and Kornatz Stegmann 1954 McQuarrie 1954) * In the absence of any well-established deficiency or excess syndrome a clearer understanding of the relationship of glucagon to human metabolism depends on demonstration of its presence in plasma and on determination of conditions causing variations in its levels

The reader is referred to the excellent reviews of Foa *et al* (1957) and de Duve and Berthet (1957) for discussion of these topics

EXTRACTION AND PURIFICATION OF GLUCAGON

In the purification of glucagon from pancreatic tissue difficulty in separation from insulin was noted by early investigators (Burger and Brandt 1935) Further some methods for crystallization of insulin do not effect a separation of glucagon (de Duve *et al*, 1946 Olsen and Klein 1947 Sutherland and Cori 1948) Thus a method designed for the quantitative extraction of insulin (Best *et al* 1939) has been used with only minor modifications for the extraction of glucagon from biological materials (Sutherland and de Duve 1948) Further purification has been achieved using various chemical fractionation techniques (Sutherland *et al* 1949 Kazal *et al* 1950 de Duve and Vuylsteke 1953a Foa *et al* 1953a) Partition chromatography has been applied to the separation of glucagon from crude insulin (Porter 1952) but not from other biological materials as yet other newer techniques such as ion exchange methods remain to be investigated With use of these extraction procedures glucagon has been obtained from pancreatic and gastrointestinal tissue of many species (de Duve and Berthet 1957 Foa *et al* 1957) including man (Kenny 1955 Makman *et al* 1958b) The application of these procedures to glucagon in plasma is discussed subsequently Staub *et al* (1953 1955) have developed a fractionation procedure for the preparation of glucagon from pancreatic tissue as a purified crystalline material thus providing a source of pure glucagon for various studies

CHEMICAL AND BIOLOGICAL PROPERTIES

The chemical properties of glucagon serve to distinguish it from a number of other hyperglycemic materials which have been obtained from the pancreas (de Duve and Berthet 1957 Foa *et al* 1957) Glucagon is not dialyzable has an isoelectric point in the range pH 7 5 to 8 5 is relatively stable in acid is more resistant to alkali than is insulin and unlike insulin is not susceptible to cysteine reduction (Sutherland and Cori 1948 Sutherland and de Duve 1958 Staub *et al* 1955) The crystalline material tends to form fibrils in acid solution (Staub *et al* 1955) The glucagon molecule consists of 29 amino acids in a single chain of known sequence with a molecular weight of 3485 (Bromer *et al* 1956 1957c) Evidence to date indicates that the entire sequence is necessary for biological activity (Bromer

et al 1957d) none of the degradation products studied including that with only a terminal amino acid removed were active No metallic or other nonprotein component appears to be part of the active material although this possibility has not been completely excluded

Glucagon is readily inactivated by the proteolytic enzymes trypsin chymotrypsin and subtilysin (Sutherland and Cori 1948 Bromer *et al* 1957a 1957b Sinn *et al* 1957) Glucagon is also destroyed by degradation systems apparently proteolytic in nature (Narahara and Williams 1957) present in a number of tissues (Kenny 1956) Degradation is particularly active in the liver (Goldner *et al* 1954 Vuylsteke and de Duve 1955a 1955b) kidneys and considering total organ mass muscle (Cox *et al* 1957 Narahara and Williams 1957) Destruction by whole blood or plasma does not appear to be significant (Kazal *et al* 1950 Kenny 1956 Narahara and Williams 1957) That the liver is not the only important site for degradation of glucagon *in vivo* is suggested by the appearance of appreciable amounts of degradation products of glucagon I^{131} in extrahepatic tissues and by the similar patterns of distribution and degradation obtained following injection of glucagon I^{131} into the portal vein and the inferior vena cava (Cox *et al* 1957) Glucagon is destroyed more rapidly than insulin on perfusion through frog liver (Goldner *et al* 1954 Weisenfeld *et al* 1957) and disappears more rapidly from the plasma of rabbits following peripheral intravenous injection of small amounts of the I^{131} labeled hormone (Berson *et al* 1957) Both the system for glucagon degradation (Kenny 1956) and the insulinase system (Mirsky and Perisutti 1957) have been reported to possess some specificity However glucagon degradation by liver slices is inhibited by insulin (Vuylsteke and de Duve 1955a 1955b) as well as by ACTH casein and growth hormone (Tybergheim *et al* 1956) suggesting that all these biological materials serve as substrates in a common inactivating system (Tomizawa and Williams 1955)

Glucagon was proposed by Kimball and Murlin (1923) as a second pancreatic hormone responsible for the hyperglycemia which these and other investigators noted shortly after the injection of pancreatic extracts (MacLeod 1922 Collip 1923 Murlin *et al* 1923) The hyperglycemia results from mobilization of liver glycogen (Burger and Kramer 1929) the degree of response being dependent on the amount of liver glycogen present The initial decrease in liver glycogen is followed by a rebound increase above control levels noted about 12 to 24 hours after glucagon administration (Costa *et al* 1956a Root

1954 1956) This rebound has been explained as the result of a secondary increase in gluconeogenesis (Kalant 1956) mediated by the adrenal cortex (Costa *et al* 1956b Galansino *et al*, 1957) *In vitro* the glucagon effect is manifested by increased glucose output from liver slices (Sutherland and Cori 1948) The effect has been shown to be related to activation of liver phosphorylase *in vivo* (Cahill *et al* 1957b) confirming *in vitro* studies with liver slices and with broken cell preparations (Sutherland and Cori 1948 1951 Sutherland 1951 Rall *et al* 1956 1957) Phosphorylase catalyzes the readily reversible reaction glycogen + inorganic phosphate = glucose 1 phosphate However an increase in active phosphorylase concentration results in glycogen breakdown rather than in synthesis Thus it appears that glycogen synthesis and degradation are somehow separated in the liver cell possibly by cellular compartments or by a pathway for glycogen synthesis other than phosphorylase such as Leloir and Cardini (1957) have recently described in hepatic tissue In this alternate pathway uridine diphosphoglucose serves as the donor of hexose units in the formation of glycogen (see Fig 1)

The relationship of glucagon and epinephrine to phosphorylase reactivation and intermediary metabolism in liver has been outlined in Figure 1 The concentration of active phosphorylase in liver represents a balance between inactivation by liver phosphorylase phosphatase and reactivation by a dephosphophosphorylase kinase system as yet only partially characterized Glucagon has been shown to act by increasing the accumulation of the active form of phosphorylase This action is not specific for glucagon epinephrine and the related amines act in a similar fashion However ergotamine and related alkaloids have been shown to block the effect of epinephrine both in the intact animal and in liver slices and broken cell preparations the effect of glucagon is not blocked (Ellis *et al* 1953 Berthet *et al* 1957) Thus the hepatic effects of these hormones can be distinguished

This effect of glucagon and epinephrine has been separated into two phases first a heat stable dialyzable factor accumulates in increased amounts when particulate fractions of liver are incubated in the presence of glucagon or epinephrine and second the factor stimulates phosphorylase activation in supernatant fractions where the hormones have no effect Recently the active factor has been isolated and identified as a cyclic adenylic acid adenosine 3 5 phosphate (Cook *et al* 1957 Lipkin Rall and Sutherland 1958 Suther

land and Rall 1957 1958) Cyclic adenylic acid once formed is rapidly inactivated by a phosphodiesterase a reaction which is in hibited in the presence of caffeine Particulate preparations from heart skeletal muscle brain kidney and chicken blood have been found to form cyclic adenylic acid under the same conditions used

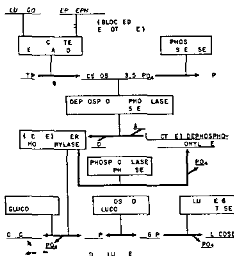


FIGURE 1 Relationship of glucagon and epinephrine to activation of liver phosphorylase and to production of glucose from glycogen This is presented in schematic form as a summary of these reactions and activating and inac tivating mechanisms insofar as present knowledge allows The dephospho-phosphorylase kinase system is complex and is influenced by inhibitors as well as activators the exact action of adenosine 3,5 phosphate (cyclic adenylic acid) in this system has not been elucidated The enzymes amylo 1 6 glucosidase and UDPG glycogen transglucosylase are not included for simplification

for the liver experiments Epinephrine stimulates heart and skeletal muscle preparations but glucagon has no effect on tissue preparations other than those from the liver Recently Haynes and Berthet (1957) have shown that the activation of adrenal phosphorylase by ACTH is mediated by an increased accumulation of cyclic adenylic acid in adrenal tissue (Haynes) Thus cyclic adenylic acid would appear to be a common mediator for the action of three hormones which cause hyperglycemia — glucagon epinephrine and ACTH Other effects of glucagon which may not be related to cyclic adenylic acid are discussed in reviews by de Duve and Berthet (1957) and Foa *et al* (1957)

BIOASSAY

Glucagon has been assayed by the degree of hyperglycemia produced *in vivo* in rabbits (Rowlinson and Lesford 1951) and with greater sensitivity and accuracy in fasted anesthetized cats (Staub and Behrens 1954) Glucagon may be assayed *in vitro* by the increase in glucose production (Sutherland and de Duve 1948 Sutherland *et al* 1949 Audy and Kerly 1952 de Duve and Vuylsteke 1953b Vuylsteke and de Duve 1957) *or in phosphorylase concentration in liver slices* (Cornblath 1955 Ui *et al* 1956) and by measurement of the rate of phosphorylase reactivation in liver homogenates (Berthet *et al* 1957) The action of epinephrine in these systems can be blocked by the presence of ergotamine as discussed previously The *in vivo* assay is the least specific and the least sensitive of these methods In the slice system in addition to glucagon and epinephrine amylase glucose precursors (Sutherland and de Duve 1948) and certain cations may influence glucose output (Cahill 1957a Hastings *et al* 1956 Vuylsteke and de Duve 1955b 1957) Insulin growth hormone ACTH and α casein inhibit glucagon degradation in liver slices thereby enhancing its effect on glucose output Thus the addition of insulin to the liver slice system results in appreciably increased sensitivity to glucagon (Vulysteke and de Duve 1957) This phenomenon must therefore be considered in any determination of the glucagon content of extracts contaminated with other proteins The homogenate assay system although requiring more difficult preparations is more specific accurate and sensitive than the other methods With the use of diluted homogenates fortified with purified liver dephosphophosphorylase and with the addition of caffeine to block the degradation of cyclic adenylic acid (see Fig 1) and sufficient insulin or casein for maximal protection of glucagon an extremely sensitive system is obtained Thus the presence of 0 001 μg of glucagon in the 0 25 ml system is readily detectable It has been estimated that an average homogenate assay involving sixty measurements of phosphorylase reactivation (Berthet *et al* 1957) or about thirty assays with rabbit liver slices involving thirty slices each (Vuylsteke and de Duve 1957) or more than two hundred measurements of the *hyperglycemic response of the anes thetized cat* (Staub and Behrens 1954) would be required to achieve equivalent degrees of accuracy

IDENTIFICATION AND MEASUREMENT OF GLUCAGON IN PLASMA

Considering the pancreas as the source of glucagon some investigators have sought to demonstrate the presence of glucagon in pancreatic blood under various conditions of possible stimulation Thus Foa *et al* (1952a 1952b 1953b) using a cross circulation procedure demonstrated a hyperglycemic response in a dog receiving pancreatic blood from a normal donor in insulin hypoglycemia or pretreated with growth hormone An effect was also noted with pancreatic blood obtained from an alloxan diabetic dog given a glucose infusion (Foa *et al* 1949) Bornstein *et al* (1951) demonstrated a hyperglycemic response in alloxan-diabetic hypophysectomized adrenalectomized (ADHA) rats receiving portal blood from ADHA rats treated with growth hormone or from intact cats with growth hormone diabetes

Bornstein obtained no response with portal blood from ADHA (Bornstein *et al* 1951) saline treated rats or with femoral blood from either control or growth hormone treated animals Foa likewise reported no response with pancreatic blood from normal or alloxan diabetic controls or with mesenteric blood from either the control or treated groups These reports do not exclude the possibility that the hyperglycemic material is present in the peripheral blood stream or in the pancreatic or portal blood of the untreated animals The use of whole blood with the possible presence of insulin and other types of interfering substances and a relatively insensitive assay system (or in the case of the ADHA rats a preparation especially sensitive to insulin) may have prevented detection of the hyperglycemic material

Some investigators have obtained hyperglycemic effects with pancreatic blood from untreated animals and with peripheral blood Fodden and Read (1955) have reported the presence of a hyperglycemic material in extracts of pancreatic blood of untreated dogs which almost tripled in amount by pretreatment with growth hormone or ACTH peripheral blood was not studied Tyberghein and Williams (1956) have reported a glycogenolytic effect of extracts of normal rabbit serum on rabbit liver slices the extracts showed the same amount of activity whether obtained from portal caval or aortic blood Burger and Klotzbucher (1947) have reported a hyperglycemic effect of human blood from normal subjects when transfused into

normal human recipients The effect was obtained only when blood was taken from a donor in a stage of alimentary hyperglycemia blood from a fasting donor produced no effect On the basis of similar experiments Klotzbucher (1953) reported that blood from fasting diabetic patients had a hyperglycemic effect in normal recipients This effect was seen in about one third of those tested another third showed a fall in blood sugar A marked hyperglycemic effect was obtained with blood from 2 patients in diabetic coma Bornstein and Lawrence (1951) reported a hyperglycemic effect when plasma from 2 diabetic *acromegalic patients was injected into ADHA rats* The relationship of the effects obtained with diabetic blood to glucagon is particularly open to question since a variety of insulin antagonists may be present in diabetic blood or appear in the circulation during diabetic acidosis (These are discussed in Chapter III and in a recent article by Berson and Yalow 1958)

Sirek (1957) and Sirek *et al* (1957) reported that pancreaticoduodenal blood from normal dogs treated with growth hormone had a hyper glycemic effect when injected into alloxan-diabetic dogs The pan creas did not appear to be the sole source of this material since a similar hyperglycemic response was produced by blood from intact pancreaticoduodenal veins of pancreatectomized dogs Further the hyperglycemic effect was blocked by dihydroergotamine a substance which blocks the action of the sympathomimetic amines but not that of glucagon These observations open to question the nature of the other hyperglycemic substances described above since possible blockade by ergotamine was not tested

In view of the complexity of regulatory mechanisms and the existence of more than a single hyperglycemic factor accurate identi fication of such factors is essential Most of the reports mentioned have dealt with effects obtained using whole blood or plasma char acterization of the causative factor involved has been limited Born stein *et al* (1951) observed their effects in alloxan diabetic animals in the absence of the adrenal and pituitary glands Further the hyperglycemic action of blood from donor rats treated with growth hormone was associated with a depletion of liver glycogen in the re cipient rats Fodden and Read (1955) using a procedure similar for the most part to those previously described for the purification of glucagon from tissues obtained plasma extracts which produced a hyperglycemic response in the cat and increased glucose output and active phosphorylase concentration of liver slices This material ap

peared to be a nondialyzable protein with an isoelectric point in the range pH 7 5 to 7 8 it was not inactivated by incubation with whole blood for 20 minutes at 37°C but did lose activity when stored in contact with alcohol or when at the alcohol ether stage (Demonstration that glucagon added to plasma behaves similarly under the conditions of their extraction procedure would be of value) The plasma extracts obtained had a specific activity equivalent to that of an amorphous glucagon sample of about 50 per cent purity This represents plasma glucagon levels equivalent to about 500 μg of crystalline glucagon per hundred milliliters for the untreated animals—surprisingly high levels in terms of the amounts necessary to produce *in vivo* responses Makman *et al* (1958) were unable to reproduce these results using the procedure described by Fodden and Read

Tyberghein and Williams (1958) described a procedure for fractionation of rabbit plasma The extracts obtained showed glycogenolytic activity in the liver slice system equivalent to 0 1 to 0 4 μg of glucagon per hundred milliliters of plasma The fractionation scheme included removal of a precipitate formed on neutralization of an acid alcohol supernate to pH 7 5 and of that portion of an alcohol ether precipitate which was insoluble at pH 3 5 Makman *et al* (1958a) have found removal of insoluble material from human or dog plasma fractions at comparable stages of fractionation results in significant loss both of endogenous activity and of added glucagon Such losses may explain in part the lower levels of activity found by Tyberghein and Williams in rabbit plasma Appreciable loss of activity of the rabbit plasma extracts and of added glucagon occurred on dialysis The dose response curves of the plasma extracts and of purified glucagon in the slice assay did not show strict parallelism this might be explained as the authors suggest by the presence of inhibitory material in the extracts Characterization of the glycogenolytic material in the rabbit plasma extracts included retention of activity in the presence of dihydroergotamine and inactivation by chymotrypsin and by a liver enzyme which inactivates glucagon

Studies conducted in our laboratory indicate that routine extraction procedures (Sutherland and de Duve 1948 Sutherland *et al* 1949) may not be adequate for the extraction of glucagon from plasma since with the use of such procedures small amounts of glucagon added to blood or to plasma could not be recovered or were recovered only in part (Makman *et al* 1958a) However with certain modifications of these procedures glucagon was recovered in high yield The

normal human recipients The effect was obtained only when blood was taken from a donor in a stage of alimentary hyperglycemia blood from a fasting donor produced no effect On the basis of similar experiments Klotzbucher (1953) reported that blood from fasting diabetic patients had a hyperglycemic effect in normal recipients This effect was seen in about one third of those tested another third showed a fall in blood sugar A marked hyperglycemic effect was obtained with blood from 2 patients in diabetic coma Bornstein and Lawrence (1951) reported a hyperglycemic effect when plasma from 2 diabetic acromegalic patients was injected into ADHA rats The relationship of the effects obtained with diabetic blood to glucagon is particularly open to question since a variety of insulin antagonists may be present in diabetic blood or appear in the circulation during diabetic acidosis (These are discussed in Chapter III and in a recent article by Berson and Yalow 1958)

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whole blood or plasma The final fraction contained on an average 71 per cent of the glucagon added to whole blood and 88 per cent of that added to plasma hence about 19 per cent was lost by re moval of the cell fraction Another 10 per cent was recovered from the acid alcohol precipitate by re-extraction Taking these losses into consideration recovery appeared to be approximately 100 per cent

With the use of this procedure a glucagon like material was demon strated in the peripheral venous blood of man and in the peripheral and pancreatic blood of the dog (Makman *et al* 1958a) in addition

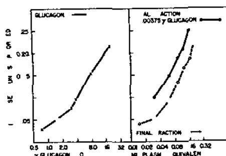


FIGURE 3 Titration of glucagon and the glucagon like material of blood in the liver homogenate assay system The amounts of glucagon plasma equivalent and increase in units LP (liver phosphorylase) are expressed as amounts present or formed per 0 25 ml which was the volume of the reaction mixture Blood was fractionated by the procedure described (see text and Fig 2) and the final fraction was assayed in the absence and in the presence of 0 00375 μg of glucagon per 0.25 ml as indicated (From Makman Mak man and Sutherland *J Biol Chem* 233 894 1958)

the material has recently been detected in the peripheral blood of kittens (Makman *et al* 1958b) The fractionation of a few milliliters of plasma was sufficient for accurate titration of the endogenous activity of the final fractions in the liver homogenate assay system Figure 3 shows the titration curves of a standard glucagon sample and a typical final fraction in the assay system These curves were parallel indicating the similarity of effects of the endogenous material and of glucagon The curve resulting from the addition of 0 00375 μg of glucagon to serial dilutions of the final fraction showed that the fraction was not inhibitory to the homogenate assay for glucagon

Figure 4 illustrates the effect on peripheral glucagon blood levels from injecting glucagon into the portal vein of an anesthetized dog Following an initial spiking the peripheral levels rapidly decreased returning to near preinjection levels in about 20 minutes This rate of disappearance of biological activity is essentially in agreement with

procedure developed is outlined in Figure 2 After removal of the cell fraction a mixture containing 15 ml of absolute ethanol and 0 3 ml of 14 normal HCl was added to each 5 ml of plasma The precipitate was removed and the supernatant fluid brought to pH 7 7 with ammonium hydroxide A modification was necessary at this point to avoid coprecipitation with plasma proteins Thus in contrast to former extraction procedures the precipitate which formed on neutralization could not be removed without considerable loss of added

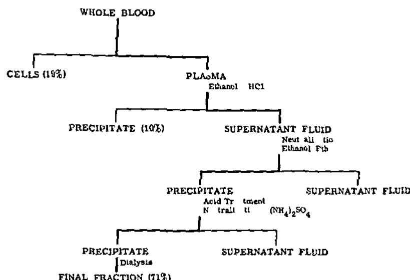


FIGURE 2 Summary of blood fractionation procedure The values in parentheses represent per cent of added glucagon (15 μg per 100 ml of whole blood) lost on removal of the cell fraction or appearing in the acid alcohol precipitate or in the final fraction

glucagon Furthermore since the alcohol ether precipitate and earlier fractions were inhibitory to the assay system a second modification was necessary to remove or inactivate these inhibitory factors Suspension and incubation of the alcohol ether precipitate in 0 135 normal HCl at 2 C for 2 hours effectively eliminated the inhibitory effect Precipitation with ammonium sulfate (42 gm per hundred milliliters of neutralized suspension) was primarily for concentration The precipitate was resuspended and dialyzed against distilled water prior to assay

This procedure was designed to achieve high recovery rather than maximal purification The final fractions were purified from five to sevenfold In order to estimate recovery samples were fractionated both with and without 15 μg of glucagon per hundred milliliters of

or in man a level of possible physiological significance This amount of circulating glucagon if free to act should elicit an appreciable hyperglycemic response Indeed the endogenous material may be doing so contributing to the balance of factors which influence the blood glucose level

After fractionation and characterization the endogenous material was in the following respects indistinguishable from glucagon added to blood Both were nondialyzable showed similar alkali stability and similar stability and solubility characteristics in a number of variations of the fractionation procedure Inactivation by trypsin and by an insulinase extract of rat liver which also inactivates glucagon indicated that the glucagon like material required an intact protein *structure for its activity Similarity of response of the homogenate assay* system to that of the final fractions in the presence or absence of ergotamine indicates that the fractions contained no detectable amount of epinephrine or its related compounds and that the endogenous material is distinct from these The final fractions also increased the output of glucose from liver slices the amount of activity was the same whether determined by the liver slice or liver homogenate assay method

Both the endogenous material and glucagon acted in the homogenate system by stimulating reactivation of phosphorylase However the endogenous glucagon like material differed from glucagon in two respects (Makman and Sutherland 1959) (1) Supernatant fractions of liver homogenates have been prepared in which the endogenous material but not glucagon increased the rate of phosphorylase activation (2) An effect such as occurs with glucagon on cyclic adenylic acid accumulation in the presence of particulate fractions of liver has not yet been demonstrated with the final plasma fractions (It is possible that some of the fractions not tested did contain material capable of acting in the particulate system which forms cyclic adenylic acid)

By these two criteria therefore the glucagon like material of blood isolated in this fractionation procedure was not identical to glucagon the response of the homogenate and slice systems could be explained by the effect on phosphorylase activation seen in the liver supernatant system alone Since the endogenous glucagon like material *is active in* slices as well as in homogenates it seems possible that this material may be active *in vivo* It should be noted that Tybergheim and Williams (1958) obtained similar results when assaying plasma fractions exclusively by the liver slice method Two major questions arise

the I^{131} tracer studies of Berson *et al* (1957) and Cox *et al* (1957) mentioned previously Thus it appeared that glucagon could be measured in peripheral blood after passage through the liver with the use of the fractionation and assay scheme described

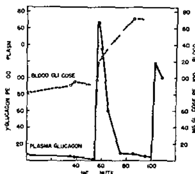


FIGURE 4 Recovery of glucagon in peripheral circulation following injection into the portal vein An 18 5 kg male dog was anesthetized with sodium seconal at zero time Four femoral venous samples were taken during a 50 minute control period At 55 minutes and again at 100 minutes 100 μg per kilogram crystalline glucagon was injected into the portal vein as indicated by arrows Femoral samples were taken at 45 seconds and at 2¼ 5 10 20 30 and 40 minutes after the first injection and at 2 and 8 minutes after the second injection Glucose levels were determined by the method of Nelson (*J Biol Chem* 153 375 1944) Plasma glucagon levels represent the total amount of active material recovered in the final fractions prepared by the procedure described (see text) and have not been corrected for loss during fractionation (From Makman Makman and Sutherland *J Biol Chem* 233 894 1958)

Close agreement has been found (Makman *et al* 1958b) in values for endogenous activity obtained by duplicate fractionation of single blood samples indicating the possible applicability of the procedure for study of variations in levels Considerable variation in plasma levels of different subjects and in one subject over a period of time has been noted both in dog and in man (Makman *et al* 1958b) The causes of such variations are being investigated at present Thus far attempts to determine the site of secretion and conditions influencing secretion have been inconclusive the origin of the endogenous material is as yet unknown Preliminary experiments have indicated that some activity is present in the plasma of the dog 1 hour after removal of the pancreas stomach and small intestine (Makman *et al* 1958b) suggesting either another site of secretion or a slow rate of inactivation of the endogenous material The average peripheral blood level of the material in terms of its crystalline glucagon equivalent is about 7μg per hundred milliliters of plasma in dog

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from these studies first, is there indeed some glucagon in plasma which has been obscured in the studies by relatively high levels of this glucagon like material and second does this glucagon like material possess significant physiological activity?

CONCLUSION

The presence of hyperglycemic materials in plasma has been indicated by a number of reports however characterization of the active material has not been extensive and studies in man have been particularly limited Our present knowledge of glucagon makes possible the development of methods for its identification and measurement in plasma A plasma fractionation procedure by which added glucagon can be recovered quantitatively and measured by bioassay has been described and its application to the study of an endogenous glucagon like material in plasma of dog and man discussed This endogenous glucagon like material was shown by various fractionation and characterization studies to be indistinguishable from glucagon However most if not all of this glucagon like material was shown to be a material other than glucagon which was capable of stimulating the activation of phosphorylase without the presence of the cyclic adenylic acid forming system Thus while a number of studies have shown hyperglycemic substances to be present in blood under various conditions the presence of glucagon itself has not been clearly demonstrated It is apparent that the variety of hyperglycemic materials which have been described can be properly compared and evaluated only after their accurate identification and measurement

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PART THREE

Hormones of Pituitary Origin

CHAPTER V

The Detection of Growth Hormone in Plasma

Ernst Knobil *and* Roy O Greep

PARABIOSIS

THE TIBIA TEST

PLASMA SULFATION FACTOR

IMMUNOLOGICAL METHODS

BIBLIOGRAPHY

Since the advent of growth hormone on the endocrinological scene some thirty six years ago impressive strides have been made toward the structural elucidation of the active molecule the characterization of its physicochemical properties and the delineation of its varied morphological and metabolic actions in vertebrates By contrast information concerning the factors which regulate the synthesis secretion and circulating levels of growth hormone is appallingly scant The filling of this hiatus is requisite for any gains in understanding of the physiology and pathophysiology of growth hormone secretion

The purpose of this brief chapter is to review the attempts which have been made to approach the problems mentioned above with especial reference to the measurement of circulating growth hormone levels as an index of adenohypophyseal secretory activity

The successful detection and quantitation of pituitary growth hormone in biological fluids rests on a suitable assay method Since adenohypophyseal hormones cannot be measured by chemical techniques at present bioassay procedures must be used Because the assay technique is the limiting factor in the reliability of any estimate of growth hormone activity in plasma or other fluids the studies reported in the literature are grouped according to the assay methods utilized

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PARABIOSIS

In an early attempt to detect circulating growth hormone and to investigate its behavior in the blood Van Dyke and his co workers (1950) devised *an elaborate and ingenious experimental* design utilizing the rat Immature animals were joined in parabiosis parabiotic pairs being variously normal normal normal hypophysectomized and hypophysectomized hypophysectomized It was found that hypophysectomized rats joined to normal ones grew appreciably more than those joined to partners which were also hypophysectomized The criterion of growth used in this study was the increase in the distance from anus to tip of tail This finding suggested that a growth promoting substance was circulating in the normal partner which crossed the parabiotic barrier and produced evidence of growth stimulation in the hypophysectomized partner This increment of growth could be duplicated in parabiotic rats both of which were hypophysectomized by injecting one of them with a daily dose of 220 μg of purified bovine growth hormone The implication of this result is that the normal pituitary gland of rats utilized in this study secreted the equivalent of approximately 220 μg of purified bovine growth hormone per day

Van Dyke *et al* (1950) further calculated that the volume of distribution of administered growth hormone *in the rat* approximates 28 per cent of the body weight and that the average life of circulating administered hormone is approximately 9 hours

THE TIBIA TEST

Of all the methods proposed for the bioassay of growth hormone the tibia test of Greenspan *et al* (1949) or various modifications thereof has been most widely used This method is based on the increase in width of the proximal epiphyseal cartilage of the tibia of hypophysectomized rats in response to growth hormone administration The following reports dealing with the assay of growth hormone in biological fluids are based on this technique

Normal Plasma

Attempts to detect growth hormone activity *in normal* lyophilized human plasma were unsuccessful in the hands of Kinsell *et al* (1948)

Assay of the globulin fraction found to contain most of the activity of added hormone also yielded negative results when obtained from growing children (Greenspan 1950) Greenspan (1950) further failed to demonstrate a significant tibial response by injecting non fractionated plasma from 54 actively growing children and 9 adults

Segaloff and co workers (1955) however assaying whole plasma of children and adults of various ages reported a growth hormone content of from 0 to 40 μg per milliliter in terms of a standard beef growth hormone preparation No correlation between the plasma content of growth hormone activity and the age of the donor was demonstrable in this study Van Dyke as quoted by Li (1950) reported that adult human plasma contains the equivalent of approximately 0 1 μg of bovine growth hormone per milliliter but no further details were given

Using the Cohn fractionation procedure Gemzell *et al* (1955a) found that bovine growth hormone added to blood could be recovered (17 8 to 28 0 per cent) in a plasma fraction equivalent to Cohn Fractions IV 1 and IV-4 The injection of this fraction into assay rats yielded values based on bovine growth hormone standards of 0 57 μg per milliliter of plasma in a young growing pig 0 37 μg per milliliter in a calf but no activity in normal human plasma

Contopoulos and Simpson (1957) using a modified tibia test body weight and tail length as criteria of growth hormone activity and whole plasma for assay concluded that the growth hormone activity of normal rat plasma is equivalent to 1 0 to 2 5 μg of purified bovine growth hormone per milliliter of plasma

Acromegaly and Gigantism

Kinsell *et al* (1948) first reported increased growth hormone activity in the lyophilized plasma of patients with gigantism and acromegaly Their data are difficult to quantitate however on the basis of the information provided in their report Greenspan (1950) could not confirm these observations in 3 patients with acromegaly using whole plasma

Gemzell *et al* (1955a) injecting appropriate plasma protein fractions into their assay rats (Cohn Fractions IV 1 and IV-4) reported in 1 patient with acromegaly a plasma content of 11 9 μg per milliliter in terms of a bovine growth hormone standard and 7 7 μg per milliliter in 1 patient with gigantism

Pregnancy

Using the globulin fraction of plasma proteins for assay Cotes and Young (1951) were unable to detect growth hormone activity in the blood of pregnant or lactating women and in one pregnant goat Urine samples were similarly without activity

Gemzell and his colleagues (1955b) assayed fractionated or lyophilized retroplacental plasma obtained from parturient women and found it to contain the equivalent (in terms of a bovine preparation) of 1 85 to 9 25 μg of growth hormone activity per milliliter In 1 patient with diabetes the retroplacental plasma contained 10 μg of growth hormone per milliliter Cord blood gave a value of 5 9 μg per milliliter These findings are in contrast to the absence of detectable growth hormone activity in the plasma of normal man studied with the same technique (see p 142)

The growth promoting activity of rat plasma is increased some threefold during pregnancy when compared to nonpregnant animals values of 3 7 to 7 5 μg of bovine growth hormone equivalents per milliliter of plasma being observed in pregnant rats (Contopoulos and Simpson 1957) This increase is not abolished by hypophysectomy (Contopoulos and Simpson 1959)

The Half life of Growth Hormone

The biological half life of bovine growth hormone in hypophysectomized rats has been investigated The plasma of such rats was assayed for growth hormone activity at intervals following a single injection of growth hormone Van Dyke *et al* (1950) calculated an average half life of approximately 26 minutes while Gemzell and his colleagues (1955b) arrived at a figure of some 40 minutes

PLASMA SULFATION FACTOR

The observation that hypophysectomy in the rat causes a reduction in the incorporation of injected sulfate S^{35} into cartilage which can be restored to normal by growth hormone administration (Ellis *et al* 1953 Denko and Bergenstal 1955 Murphy *et al* 1956) led to the finding by Salmon and Daughaday (1957) of a factor present in plasma which stimulates the uptake of sulfate *in vitro* by cartilage excised from hypophysectomized rats This sulfation factor in rat plasma disappears after hypophysectomy and its presence can be restored to

normal levels following administration of bovine growth hormone in physiological quantities The sulfation factor is not growth hormone itself since growth hormone when added to the medium *in vitro* does not promote sulfate uptake by cartilage from hypophysectomized rats While the nature of the sulfation factor is still obscure its presence in the plasma appears to be dependent on circulating growth hormone Daughaday and co workers (1958 1959) have further shown that normal human plasma contains sulfation factor which does not vary significantly with the age of the donors Reduced sulfation factor activities were observed in hypophysectomized patients and in patients with pituitary dwarfism and post partum necrosis Elevated values were found in acromegaly Preliminary studies (Daughaday 1958) indicate that the administration of small doses of human and monkey pituitary growth hormone to hypopituitary subjects effected a highly significant increase in plasma sulfation factor activity

IMMUNOLOGICAL METHODS

A highly purified preparation of bovine growth hormone was found to be antigenic in the rabbit by Morrison *et al* (1952) The specificity of this reaction was not established however at least as far as other adenohypophyseal hormones are concerned Using the more sensitive tannic acid hemagglutination test (Boyden 1951) Ferguson and Boyden (1953) were able to detect antibodies reacting with a purified preparation of bovine growth hormone in the sera of sheep following injection of crude anterior pituitary extracts or of purified bovine growth hormone This reaction appeared to be specific for growth hormone since absorption of the sera with pituitary preparations containing adenohypophyseal principles other than growth hormone or with ox serum did not inhibit agglutination The antigenicity of highly purified growth hormone has also been investigated by Hayashida and Li (1958) by means of anaphylactic shock experiments in guinea pigs precipitin ring tests with rabbit antisera and bioassay of the antiserum in hypophysectomized rats for antiserum activity These workers were able to detect as little as 1 μg of growth hormone by the precipitin test Rabbit antiserum did not react with any of the other pituitary hormones at the concentrations studied attesting to the specificity of the reaction

Read and his collaborators (1958 1960) have applied the immunological properties of growth hormone to an assay for this hormone in

human blood This method is based on the tannic acid hemagglutination test of Boyden (1951) The procedure is summarized as follows

In the first stage of the assay sheep erythrocytes are treated with dilute solutions of tannic acid the cells are washed and then exposed to a solution of a highly purified human growth hormone preparation Following further washing they are added to serial dilutions of sera taken from rabbits previously immunized against the human growth hormone (antigrowth hormone serum) The highest titer of antiserum which agglutinates the antigen treated erythrocytes is determined

In the second stage of the assay decreasing known concentrations of human growth hormone are added to the appropriate dilution of rabbit antiserum The growth hormone inhibits the activity of homologous antiserum Hormone treated sheep erythrocytes are thus agglutinated only in the tubes which do not contain enough growth hormone to inactivate all of the antibody The minimal amount of growth hormone with which the rabbit antiserum reacts can thus be determined

In the third stage serial dilutions of human serum are substituted for the known growth hormone dilutions That dilution of serum which first inhibits hemagglutination is thus established This dilution contains the same amount of growth hormone which inhibits hemagglutination in the second stage of the procedure The concentration of hormone in the serum can then be calculated This method appears to be specific for growth hormone and is very sensitive permitting the detection of fractions of a microgram of growth hormone Using this technique Read (1960) states that in normal children and adults the levels of growth hormone in serum range between 0 16 and 0 64 μg per milliliter

The development of immunological techniques for the assay of growth hormone in biological fluids provides a major advance in this field The preliminary information available at this time suggests that such methods possess the sensitivity and specificity necessary for reliable determinations In this the immunological technique far surpasses the tibia test which at best has not been very useful in the detection of circulating growth hormone principally because of its shortcomings in the area of specificity (Geschwind and Li 1955)

If the proposed immunological methods for the assay of growth hormone live up to their high promise they will provide for the first time the means for a long-delayed attack on problems dealing with the regulation of growth hormone secretion and metabolism

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CHAPTER VI

Adrenocorticotropic Hormone

Paul L Munson

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- Concept of a continuous basal secretion of ACTH
- Increased secretion of ACTH stress and loss of adrenal glands

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INTRODUCTION

Assay of ACTH in Plasma, Limitations of Available Methods

The amino acid sequence of several polypeptides with adreno corticotropic hormone (ACTH) activity isolated from pituitary tissue is now known with reasonable completeness (Shepherd *et al* 1956a b) and no structural feature sufficiently unique to serve as the basis of a specific chemical analytical method has come to light For the present and foreseeable future therefore estimation of the concentration of ACTH in biological material including human plasma must depend on biological assay

The present methods of biological assay for ACTH of which there are several basic types are far from adequate for the satisfactory quantitative estimation of ACTH in blood The principal difficulty is the disparity between the low concentration of ACTH in circulating blood and the relatively high dose requirement of the assay methods a much more delicate method is needed However the inadequacies of present methods have not precluded the accumulation of a con siderable body of informative data

There are numerous indirect measurements from which the level of circulating ACTH in subjects with intact adrenal glands may be inferred such as for example the concentration of cortical steroids in the plasma and the amount of the steroids and/or their metabolites excreted in the urine However factors other than ACTH affect the levels of cortical steroids in blood and urine Among them are the extent and nature of conjugation and other metabolic transformations

of the steroids in liver and other tissues the rate of excretion of the steroids and the sensitivity of the adrenal cortex to ACTH all of which may vary from subject to subject and from time to time The measurements of steroid levels in body fluids and the responses of the target tissues are significant in themselves but for the definitive determination of ACTH in blood direct methods are essential As these methods become more precise more reliable and more feasible they will assume increasing importance for certain problems of diagnosis for evaluation of therapy and for investigation of basic mechanisms In patients and animals without functional adrenal glands the endogenous adrenal steroid level obviously zero cannot serve as an indirect indication of the level of circulating ACTH Here as in intact subjects quantitative estimation of plasma ACTH under various conditions by a direct method will contribute impor tantly to a better understanding of the physiology of the anterior lobe of the pituitary gland and hypothalamus

In addition to the usual desirable attributes of a biological assay method specificity precision convenience and economy a *low minimum effective dose* (MED) because of the small concentration of ACTH in blood is an essential requirement for the bioassay of ACTH in blood

Only relatively small amounts of blood may conveniently and justifiably be collected from a human subject particularly when he may be in poor physical condition such as in Addison s disease The demands of some investigations for repeated blood samples from the same subject place a further limitation on the maximum permis sible volume of blood drawn at each venesection both by introducing extraneous variables into the study and by jeopardizing the general welfare of the patient Even if a large volume of blood can be made available the test animal cannot tolerate the rapid injection of excessively large volumes of plasma in the case of the hypophysec tomized rat certainly no more than 4 ml per hundred grams of body weight The injection of ACTH in a large volume of plasma necessitating a slower rate of injection may not cause as large an effect in the test animal as the same amount of ACTH in a small volume that can be injected rapidly Certainly the rate of injection of the ACTH is an important factor to be considered in control of the assay

The ideal method of bioassay of ACTH in plasma must be *sensitive* as well as *delicate* if one of the principal criteria of a reliable assay

is to be met namely the absence of nonparallelism in response between test sample and reference standard Obviously the test for nonparallelism requires that at least two dose levels of both unknown and standard be administered Somewhat less obvious but equally important is the requirement that all dose levels must be administered in replicate to groups of test animals so that quantitative information about animal variation is provided Otherwise no base is available either for evaluation of the possible nonparallelism or for computation of the confidence limits of the estimate of ACTH potency The ideal assay includes at least three dose levels so that the data may be tested for nonlinearity as well as for nonparallelism The use of two or three dose levels in the assay and of groups rather than single animals at each dose level further increases the amount of plasma required for the assay On the other hand the more sensitive the assay method that is the more responsive it is to small increments in dose the less the total amount of plasma required

The dose of plasma equivalent that can be conveniently and effectively administered to the test animal assuming that a sufficient amount of plasma is available could be increased if the ACTH of the plasma could be concentrated essentially quantitatively in a single plasma fraction containing only a small proportion of the total plasma solids Some progress has been made in this direction by Bethune *et al* (1958) using the standard fractionation schemes of Cohn *et al* (1946) and Oncley *et al* (1949) More drastic fractionation procedures have been employed with varying success by other investigators The data in the literature are still too meager to recommend any one of the methods unreservedly Obviously more investigation of this problem is needed and it in turn will be greatly facilitated by the availability of more delicate and sensitive bioassay methods

The foregoing discussion has been based on the assumption that the concentration of ACTH in the blood is truly very small an assumption that is not in accord with the observations of all investigators in this still difficult and controversial field The work of those who have reported relatively high ACTH concentrations in human plasma is considered later in the chapter (p 169)

Concept of a Continuous Basal Secretion of ACTH

The usefulness of determinations of ACTH in human blood or plasma does not depend on the correctness of any hypothesis that there is a normal concentration of ACTH which rises and falls only

under special or pathological conditions The concept that the normal size and function of the adrenal cortex depend on a more or less continuous secretion of ACTH by the anterior pituitary gland derives from the common observation that following hypophy sectomy of experimental animals the adrenal cortex involutes grossly the zona fasciculata and zona reticularis deteriorate and the rate of secretion of cortisol and/or corticosterone diminishes markedly Whether or not the adrenal cortex selectively binds ACTH as sug gested by the data of Sonenberg *et al* (1951) or merely depends on a certain environmental (blood) level of ACTH the further and much more tenuous concept has arisen that there is a rather low basal level of ACTH in the blood that is sufficient to maintain a healthy adrenal cortex morphologically and functionally characteristic for the species the strain the sex the age the individual and the condi tion of life This rather fuzzy and elusive concept is rendered even shakier by the fact of diurnal variation in adrenal function which is probably due to a diurnal pattern in rate of ACTH secretion rather than in adrenal cortical sensitivity to ACTH (Migeon *et al* 1956 Perkoff *et al* 1959) The further illumination of the concept must await assays of blood for ACTH by more delicate and sensitive methods For the present the constancy or inconstancy of the real or imaginary basal level of blood ACTH is masked by undetecta bility or inadequate quantitation

The very rapid rate of disappearance of ACTH (the biological half life in rats has been estimated to be one minute Sydnor and Sayers 1953) if coupled with the demonstration of a relatively constant presence of ACTH in the blood will support the concept of continuous rather than intermittent secretion of ACTH For investigation of the normal level of ACTH in blood it is obvious that the conditions under which blood samples are collected must be controlled so that an increase in ACTH secretion is not provoked by the very process of obtaining a sample

Increased Secretion of ACTH Stress and Loss of Adrenal Glands

Certain types of stress lead to an increased rate of secretion of ACTH barely sufficient to be detected at least in intact animals by available methods of assay Adrenalectomy results in an increased level of circulating ACTH usually ascribed to the marked fall in the concentration of cortisol or corticosterone which normally exerts an inhibitory influence on the secretion of ACTH by a direct action on

the anterior pituitary gland and/or indirectly by affecting the hypothalamic region *responsible for the elaboration of the hypotha*lamic secretory factor for ACTH (SF ACTH) in stress The adrenalectomized rat has been a particularly useful test animal because even with present assay methods the level of blood ACTH is sufficiently high to permit the study of the effect of various treatments

METHODS OF BIOLOGICAL ASSAY

There are several biological assay methods that have proved to be extremely useful in the estimation of the ACTH content of pituitary tissue and of purified preparations obtained by fractionation of pituitary extracts The hypophysectomized rat adrenal ascorbic acid method of M A Sayers *et al* (1948) the method of Nelson and Hume (1955) *in which the adrenal venous 17 hydroxycorticosteroids of the hypoph*ysectomized dog are measured and the somewhat more controversial method involving the release of steroids by the rat adrenal *in vitro* (Saffran and Schally 1955) are well known and have been put to intensive use Unfortunately the dose requirement of all three assay methods and of all other methods as yet generally available is too high for the convenient and reliable quantitative assay of ACTH in human plasma However there are at least two new as yet unpublished methods *with considerably lower MED s that give some promise for* the future (Lipscomb and Nelson 1959 Munson *et al* 1959)

A full description and critical analysis of all methods used for the bioassay of ACTH would be beyond the scope of this chapter What will be done is to present the bioassay methods that have been used by various authors to demonstrate the presence of ACTH in human plasma and to attempt to make quantitative estimations of the actual concentrations present under differing conditions

Adrenal Ascorbic Acid in Rats

Classic Procedure in Hypophysectomized Rats The discovery by G Sayers *et al* (1946) that administration of ACTH to the rat results in a rapid and marked fall in adrenal ascorbic acid was soon followed by the astute application of this observation to the development of a relatively simple and adequately quantitative biological assay method (M A Sayers *et al* 1948) It would be difficult to overestimate the importance of this method as a stimulant to progress in the understanding and use of ACTH The availability of the method in early

1946 undoubtedly was a large factor in making it possible for Munson and co workers at the Armour Laboratories in 1947–1948 to prepare a sufficient quantity of standardized active preparations for distribu tion to scores of investigators who thus were enabled to discover both in the clinic and the laboratory many interesting new properties of the hormone This assay method also contributed heavily to the development of commercial methods of preparation of ACTH for clinical use and to the eventual purification of the hormone A slight modification of the original assay method (Munson *et al* 1948) forms the basis for the present official method in the United States and several other countries Because the Sayers method is a relatively deli cate method it has also provided a large share of the total information we have to date on ACTH concentration in human plasma

The test animals are young rats hypophysectomized 18 to 30 hours previously Hypophysectomy eliminates interference in the assay from the rat s own ACTH that may be secreted in excess in response to the stress of handling anesthesia and injection or from pharmacologically active contaminants in the ACTH preparation The potent stress of surgical trauma was also involved in the original assay procedure in which one adrenal gland to serve as a control is removed for analysis just before the injection The injection is made intravenously and rapidly One hour later the remaining adrenal gland is removed and analyzed for ascorbic acid The difference in ascorbic acid concen trations of the two adrenal glands is taken as the response It was recommended that the standard (originally an ACTH product pre pared by Munson in 1944 and denoted La 1 A [Munson *et al* 1950] later adopted as the International Standard) and the unknown each be administered at three dose levels to groups of 6 or more rats in order to provide adequate statistical tests for nonparallelism and non linearity The potency of the unknown and its error were computed by standard statistical procedures

Modification Adopted for U S Pharmacopeia Several modifications in the assay procedure were introduced by Munson *et al* (1948) and it is in the form of the so-called Munson modification that the method appears in the U S Pharmacopeia The only important modification was to eliminate the removal of one adrenal gland just before injec tion of ACTH In the modified procedure both adrenal glands were removed one hour after injection In the hands of Munson and his collaborators this simplification facilitated the conduct of the assay in routine use without loss of precision This was also the experience

of many of the laboratories collaborating in the development of the official assay method and was influential in the decision to adopt the Munson modification for the U S P In the paper by M A Sayers *et al* (1948) a comparison between the two procedures that was interpreted to reflect adversely on the Munson modification was reported The mean index of precision (λ) reported for the original Sayers procedure in 7 assays (92 rats) was 0 176 When the same assays were recalculated without use of control adrenal glands λ was 0 325 However 4 separate assays (46 rats) conducted according to the Munson modification yielded a mean λ of 0 205 It was tentatively concluded by the authors that the modified procedure while somewhat more convenient was so much less precise that in order to attain the same standard error of a potency estimate approximately two times as many test animals would be needed In fact however the amount of data provided was inadequate for a firm conclusion The experience (unpublished) of Munson and his collaborators involving several hundred ACTH assays by the modified method in general confirms the report by M A Sayers *et al* concerning the numerical value of the index of precision (0 205) but supports the more usual observation that the indices of precision of the two procedures are not significantly different

As a general recommendation for an ACTH assay by the adrenal ascorbic acid method a rather large number of rats should be used distributing them efficiently between groups for standard and one or more unknowns according to the minimum error table of Sheps and Hendrie (1958) If the original Sayers procedure involving the removal of the control adrenal gland before injection can be followed without reducing the total number of rats in the assay this is to be preferred By so doing the results may be computed both with and without use of the control values and the more precise of the two sets of values may justifiably be used for computation of the results However if omission of separate removal of the control adrenal glands permits the use of a larger number of animals in the assay the Munson modification is to be preferred

In further application of the Munson modification it has been found (Munson 1958 Rerup 1958) that the assay is as precise if only one adrenal gland from each rat is removed and analyzed Furthermore when control values are needed such as in the detection of ACTH at very low doses (for example in plasma samples) either a *separate control group* may be added or control values may be made

up from a small number of adrenal glands taken from treated rats just before injection

Procedure in Intact Rats "Blocked" by Cortisol The method of Hodges and Vernikos (1959) is essentially identical to the method of M A Sayers *et al* (1948) except that intact rather than hypophysectomized rats are used The rats are pretreated with cortisol which apparently suppresses the normal stress response to ether anesthesia unilateral adrenalectomy and intravenous injection of rat blood so effectively that there is no interference in the assay from ACTH secreted by the test rat s own pituitary gland Confirmation of these observations from other laboratories is awaited with much interest because the substitution of intact for hypophysectomized rats would certainly facilitate the more widespread use of the adrenal ascorbic acid bioassay method Application of the method of Hodges and Vernikos to the assay of human plasma has not yet been reported

Adrenal Venous 17 Hydroxycorticosteroids in Hypophysectomized Dogs

The second biological assay method that has contributed importantly to our present stock of information about the concentration of ACTH in human plasma was developed by Nelson and Hume (1955) In this method a dog is hypophysectomized and a cannula (with bypass) is placed in an adrenal vein and exteriorized The following day a series of standard and unknown ACTH samples are administered by the intravenous route at intervals of approximately 20 minutes Within a few minutes after each injection a sample of adrenal venous blood is collected for 5 minutes by temporarily closing the bypass with a string ligature (The original paper should be consulted for additional technical details) An appropriately prepared and purified extract of the plasma is analyzed for 17 hydroxycorticosteroids by the colorimetric procedure of Nelson and Samuels (1952) and an estimate of the ACTH potency of the unknown which may be human plasma may be made statistically by a comparison of the responses to the standard and unknown respectively

One of the more attractive features of this method is the use of increased secretion of adrenal cortical steroids the principal function of ACTH as the assay response In contrast the adrenal ascorbic acid method is based on an effect that has not yet been shown to have any intrinsic physiological or biochemical importance Another advantage of the method of Nelson and Hume (1955) is that a number of comparable measurements including the responses to both the standard

and unknown may be made in the same test animal thus tending to reduce biological variation In fact at least in the hands of the originators of the method a high order of precision has been attained The mean index of precision (λ) was stated to be 0 12

Unfortunately the method has serious limitations for the assay of ACTH in human plasma The MED approximately 1 to 2 milliunits is four to eight times higher than that of the adrenal ascorbic acid method Although this difficulty is partially overcome by the superior precision of the method which thus requires a smaller number of replicate injections for a specified standard error the amount of plasma required even from a patient whose plasma ACTH level is elevated is usually too large to permit more than one injection of each sample The precision of the potency estimate is correspondingly low and it is impossible to compute confidence limits for individual plasma samples The difficulty can be evaded only by averaging the results from groups of similar patients or by repetitive assays of pooled plasma Probably because of this problem assay of the plasma at more than one dose level has not been reported

Plasma Corticosterone in Hypophysectomized Rats

The recent development of a convenient and apparently reliable fluorometric method for corticosterone by Silber *et al* (1958) has made it feasible to develop assay methods for ACTH based on the increased secretion of the major adrenal cortical steroid in rats In the method of Guillemin *et al* (1958) the ACTH is injected intravenously into rats hypophysectomized 24 hours previously Fifteen minutes later a blood sample is collected and the corticosterone content is estimated Unfortunately only a slight improvement in delicacy over the adrenal ascorbic acid method was observed The MED on the linear portion of the log dose response curve was stated to be 0 1 milliunit which is only about one half that for the classic adrenal ascorbic acid method (0 2 to 0 25 milliunit) In the report by Guillemin *et al* (1958) 0 4 milliunit is given as the MED for the adrenal ascorbic acid method This figure however was for rats weighing 160 to 210 gm and although in agreement with the MED of 0 2 to 0 25 milliunit per hundred grams found by Sayers and others it neglects the fact that rats weighing as little as 100 gm are entirely suitable for an adrenal ascorbic acid assay It is possible that in rats weighing only 100 gm which should also be quite suitable for the corticosterone assay the

useful MED may be only 0 05 milliunit but this remains to be demonstrated

Adrenal Venous Corticosterone in Hypophysectomized Rats

A logical extension of the observations of Guillemin *et al* (1958) was investigated by Lipscomb and Nelson (1959) Two modifications of the assay procedure have been developed In the first the ACTH is injected into the jugular vein of 200 gm female rats hypophysecto mized 24 hours previously Five minutes later collection of adrenal venous blood is begun with heparinized syringe and needle as de scribed by Munson and Toepel (1958) A total of 0 6 ml of blood is collected which requires 2 to 4 minutes and 0 2 ml of the plasma is used for fluorometric estimation of corticosterone The MED was 0 01 milliunit of ACTH (per rat) the log dose response curve was linear over the range 0 01 to 1 0 milliunit and the mean index of precision (λ) in 9 experiments with U S P standard ACTH was 0 18 (range 0 01 to 0 50)

In the second modification the ACTH is administered by retrograde injection up the adrenal vein and into the adrenal gland The ACTH is held in the adrenal gland for 90 seconds by occlusion of the adrenal vein with the needle used for injection (and collection) Adrenal venous blood is then collected as before except that a shorter time interval (90 seconds rather than 5 minutes) is allowed to elapse between injection of the ACTH and collection of adrenal venous blood The MED was demonstrated to be not more than 0 001 milli unit of ACTH and the log dose response curve although somewhat heteroscedastic was linear from 0 001 to 0 5 milliunit Unfortunately the slope was flatter than in the first procedure so with no apparent change in variability of response between rats the precision suffered The mean index of precision in 13 experiments was 0 47 (range 0 22 to 0 84) a serious handicap to precise bioassays with small numbers of test animals It is hoped however that further perfection of the method may improve the precision A number of samples of plasma from patients with Addison s disease were tested by this second method and the results were stated to agree satisfactorily with the results obtained on the same samples assayed in the hypophysecto mized dog by the method of Nelson and Hume An experiment in which standard ACTH was added to normal human plasma (obtained from outdated bank blood from which native ACTH had presumably

disappeared) and in which the mixture was assayed in comparison with standard ACTH alone demonstrated no significant enhancement or inhibition of the effect of the added ACTH

Adrenal Venous Ascorbic Acid in Hypophysectomized Rats

Briggs Toepel and Munson (Munson and Briggs 1955 Briggs and Toepel 1958) investigated the effect of ACTH on adrenal venous ascorbic acid with the original objective of determining the fate of the ascorbic acid lost from the adrenal gland following ACTH administration They found that ACTH did cause an increase in adrenal venous ascorbic acid that was nearly equal to or perhaps even greater than the fall in adrenal ascorbic acid (Munson and Toepel 1958) Similar results were obtained by Slusher and Roberts (1957) In the process of studying the phenomenon further Munson and Toepel (1958) found that the effect on adrenal venous ascorbic acid was linearly related to the log of the intravenous dose with an MED of 0 01 to 0 02 milliunit per100 gm suggesting a new biological assay method The results were obtained in plateaued female rats weighing about 250 gm thus giving an actual MED of 0 025 to 0 05 milliunit per rat The method of cannulation although effective was cumbersome technically extremely exacting and applicable only to rats with rather large adrenal veins

It was subsequently possible to refine the procedure for collection of blood using an ordinary syringe and needle so that the technical difficulty of this part of the procedure was reduced or eliminated The needle attached to a 1 ml syringe was inserted into the renal vein at which time the dose of ACTH could optionally be injected intravenously Within a few seconds thereafter the needle could be threaded up into the adrenal vein without removal from the renal vein It was possible to begin collection of the adrenal venous blood within about 15 seconds after completion of the injection which greatly facilitated studies of the time course of the reaction Collection of the blood was effected by careful manipulation of the plunger of the syringe guided by observation through a dissecting microscope Sufficient adrenal venous blood may be collected in about 3 minutes for the ascorbic acid analysis by a micromodification of the method of Roe and Kuether (1943) The new technique was readily applicable to a wide variety of rats including rats as small as 150 gm in body weight The method for collection of adrenal venous blood was adopted by

Lipscomb and Nelson (1959) and contributed to the development of their new biological assay methods (p 159)

The new method has made it possible to assay the unprocessed blood of adrenalectomized rats for ACTH at two dose levels in comparison with standards thus providing one important type of evidence for the specificity of measurements of ACTH in blood lack of nonparallelism between standard and unknown Similar tests on plasma from patients with Addison s disease are still in a preliminary stage

In general the MED for this assay method is higher and the responses tend to be more variable in male rats of all ages and in young female rats than in female rats 3½ months or older An extensive study of one particular group of female rats demonstrated a gradual increase in the magnitude of the response to a standard dose of ACTH (0 1 milliunit per hundred grams) as the rats aged reaching a plateau of an increase of 300 per cent over systemic blood at the age of 3½ months and continuing at that level until the age of 6 months had been attained when the experiment was terminated Other groups of female rats at the indicated optimal age have responded similarly Even assuming that the dose per rat is more important than the dose per hundred grams of body weight the response is greater in the older female rats

In spite of the low MED relative to that in the classic method of M A Sayers *et al* (1948) the dose requirement for the method of Munson and Toepel is still too large for the assay of low concentrations of ACTH in plasma without concentration of the activity by some type of fractionation For example with an MED of 0 025 milliunit of ACTH per hundred grams in a typical assay rat weighing 200 gm using two dose levels 0 05 and 0 1 milliunit of ACTH per rat with four rats to a dose level a total of 0 6 milliunit would be required for an assay the amount present in 60 to 300 ml of normal plasma based on the results of Fujita (1957) and Bethune *et al* (1958) respectively Further study and resulting modifications in the procedure however may overcome this limitation

Rat Adrenal Corticosteroidogenesis *in Vitro*

In the continuing search for more and more delicate biological assay methods for ACTH it was to have been expected that attention should turn early to the use of a system in which the administered

ACTH was applied directly to the target tissue *in vitro,* thus obviating dilution of the dose in the general circulation The pioneering studies of Hechter and associates (reviewed by Hechter and Pincus 1954) demonstrated clearly that ACTH was effective in promoting cortico steroidogenesis when perfused directly through the isolated adrenal gland Subsequently Saffran *et al* (1952) showed that an increased amount of steroid appeared in the medium in which rat adrenal slices were incubated when ACTH was added Saffran and co workers (Saffran and Bayliss 1953 Saffran and Schally 1955) went on to develop a biological assay method based on this observation It might have been assumed that a method in which the ACTH is applied directly to the adrenal cortex would require much less of the hormone than when it is given by one of the usual routes However even in this system the MED of ACTH was found to be disappointingly high about 2 to 4 milliunits per incubation flask containing quartered rat adrenal glands and several vessels are required for a proper assay For this reason alone the method is unsuitable for the assay of ACTH in human plasma and in fact there are no published reports of successful attempts to assay ACTH in plasma by the *in vitro* adrenal system In addition it has been shown by Roberts (1957) and in part confirmed by Kitay *et al* (1959) that plasma and plasma fractions from hypophysectomized rats provoke an apparent nonspecific re sponse in the system In general defense of the assay method as applied to crude ACTH preparations other than plasma it has been pointed out that the plasma proteins do not give a dose response curve and therefore could not be mistaken for ACTH in properly conducted assays (Kitay *et al* 1959) Nevertheless for the present at least no *in vitro* biological assay system that is suitable for the assay of ACTH in plasma is available

Melanocyte stimulating Activity

Even after extensive purification ACTH polypeptides retain some melanocyte stimulating activity although of a much lower order than that of pure melanocyte stimulating hormone (MSH) From the close similarity of the sequence of amino acids in pure MSH to the N terminal 13 amino acids of pure ACTH demonstrated (and re viewed) by Harris and Lerner (1957) it is reasonable to assume that MSH activity is an intrinsic property of ACTH Although these facts were unknown to Sulman in 1952 it had already been claimed that there was a high correlation between ACTH and MSH activities in

a number of partially purified ACTH preparations and that there was a considerable parallelism between the level of secretion of MSH and of ACTH in a variety of conditions dramatically so in Addison s disease These considerations led Sulman (1952) to describe and pro pose a so-called bioassay method for ACTH based on a response associated with MSH activity the darkening of the skin of the frog Now that more information is available about the chemical separa bility of the two hormones and the differences in their chemical structure and biological properties there is no longer any justification for the use of an assay for MSH as a quantitative assay for ACTH and there would not be even if there were a perfect correlation between the levels of MSH and ACTH in plasma by no means a demonstrated fact (Halkier 1953) The literature concerned with the exploration and application of the biological assay method of Sulman is therefore not included in this review

METHODS OF CONCENTRATION AND FRACTIONATION OF ACTH IN HUMAN PLASMA

As summarized in a later section (p 166) several careful investi gators have been unable to detect ACTH in normal human plasma by injecting the untreated plasma into the test animal Even in the case of plasma from patients with Addison s disease in which as almost all investigators agree the plasma ACTH concentration is usually markedly elevated the results obtained from the injection of plasma itself in volumes tolerated by the hypophysectomized rat have often been uncomfortably close to the MED of the adrenal ascorbic acid bioassay method employed Two methods known to be useful in the preliminary purification of ACTH from pituitary tissue have therefore been adapted to human blood or plasma in order to make it possible to administer to a test animal an amount of concentrated extract equivalent to a volume of plasma too large to be tolerated otherwise

Acid Acetone Extraction and Precipitation

The method of Lyons (1937) in which ACTH is extracted with acid acetone at a concentration of 80 per cent and subsequently precipitated by increasing the acetone concentration to approximately 90 per cent was apparently first applied to plasma by Bornstein and Trewhella (1950) No tests for quantitative recovery were made by

these authors However in later work their colleagues Gray and Parrott (1953) did conduct an extensive series of recovery experiments using both the original procedure of Bornstein and Trewhella and a slight modification of this procedure introduced by the authors themselves Gray and Parrott at first found that there was an apparent quantitative recovery of added ACTH by the method of Bornstein and Trewhella assuming a relatively insignificant amount of ACTH in the plasma By the authors modified procedure however in 20 out of the 29 plasma samples used recovery was greater than 100 per cent (It was just 100 per cent in the remaining 9 samples) The authors then concluded that there was sufficient ACTH originally present in these 20 plasma samples to become detectable once the total concentration had been lifted well above the MED of the bio assay method Failure to observe greater than 100 per cent recovery by the method of Bornstein and Trewhella was attributed to loss of ACTH in the original acid acetone procedure

In the experience of Sydnor and Sayers (1952b) of Paris *et al* (1954) and of Montanari *et al* (1951) the acid acetone procedure did not recover the ACTH activity of blood or plasma from adrenalectomized rats or Addisonian human patients quantitatively It is certainly true that extraction of hog pituitary tissue with 80 per cent acid acetone removes only a fraction of the total ACTH activity from the tissue (Munson and Naylor unpublished) while Montanari *et al* (1953) found ACTH activity in the supernatant fraction after precipitation of the acid acetone extract of normal human plasma at an acetone concentration of 94 per cent

Adsorption on and Elution from Oxycel

Sydnor and Sayers (1952a b) were the first to apply the Oxycel adsorption and elution technique of Payne *et al* (1950) to the concentration of ACTH in blood (In practice the modification of Bartholomew [1953] was used) They reported two experiments testing the recovery of respectively exogeneous and endogeneous ACTH from rat blood by this method In the first experiment standard ACTH was added to the blood of hypophysectomized rats The Oxycel extract of the mixture was then assayed in comparison with the ACTH alone at three dose levels and recovery of 82 ± 12 per cent was obtained In the second experiment the Oxycel extract of blood collected from adrenalectomized rats under ether anesthesia was assayed in comparison with the same blood untreated The authors

concluded that a recovery of at least 70% was obtained These results suggest that the Oxycel technique effects a reasonably quantitative recovery of ACTH from rat blood The estimates of recovery however are subject to a not inconsiderable error and are not necessarily correct for human plasma Parrott (1955) used the Oxycel technique for the further purification of acid acetone extracts of human plasma In her hands recovery of added ACTH was rather poor only 40 per cent Fujita (1957) with the same Oxycel technique as that used by Sydnor and Sayers estimated the normal ACTH content of human blood to be 1 milliunit per liter a figure considerably lower than that indicated by the work of Bethune *et al* (1958) who injected untreated plasma as well as the relatively astronomical values assigned by Bornstein and Trewhella (1950) Montanari *et al* (1951) and others

Ethanol Precipitation

The studies of Bethune *et al* (1958) were primarily designed to determine the plasma protein fraction in which ACTH is carried but may also possibly point the way to a feasible method of concentration of plasma ACTH They found the bulk of the activity in Cohn Fraction II + III with additional amounts appearing but not with perfect consistency in Fractions IV 1 and IV 4 The fractions were tested in the hypophysectomized dog by the method of Nelson and Hume (1955) and averaged about 1 0 milliunit of ACTH per hundred milliliters of plasma

DESIGN OF BIOLOGICAL ASSAYS FOR ACTH IMPORTANCE OF TWO OR MORE DOSE LEVELS

Essentially the complete body of published data on the ACTH concentration of human plasma is based on tests a more appropriate term than assays involving only one dose level of each plasma sample examined An important piece of evidence that the plasma constituent responsible for the biological response in the assay method is truly ACTH is therefore missing It is the demonstration that no statistically significant nonparallelism exists between ACTH administered in plasma or plasma extract and standard ACTH a demonstration that requires the comparison of two log dose response lines based on two or more dose levels each of both unknown and standard Such assay experiments in which two dose levels of

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both standard and unknown are included cannot prove that the response lines are parallel but they can provide a test for non parallelism relative to the error of the assay Three dose assays pro vide an even more sensitive test of identity because they may be used to test for nonlinearity of response against log dose of the presumed ACTH in the plasma as well as for nonparallelism Consistent demon stration of a lack of nonparallelism when presumptive ACTH in plasma is tested against standard ACTH will not prove that the plasma constituent is truly ACTH but it will expose its identity to a critical challenge

There are fairly obvious explanations for the absence of data on plasma ACTH tested at more than one dose level In many cases the dose of plasma or plasma extract required to produce a minimally significant response was at or near the maximum dose tolerated by the test animal The amount of plasma available was another limi tation particularly when the source was an ill or debilitated patient Pools of plasma have been collected by several investigators to obviate this difficulty a procedure that does not answer the needs of individual patients or of groups of patients with rare disorders In most bioassay methods particularly the most widely used that of M A Sayers *et al* (1948) the high dose must be three to four times the low do e in order to insure a significant regression when small numbers of test animals are used a requirement further increasing the quantity of plasma needed and placing an additional strain on the tolerance of the test animal

Solution of this urgent problem lies in the development of more delicate bioassay methods and there appears to be some hope that they will be forthcoming

ACTH LEVELS IN NORMAL HUMAN PLASMA

Negative Tests

The first well documented test for ACTH in human plasma by a specific biological assay method was by G Sayers *et al* (1949) and no ACTH was detected The tests were part of a study of the effects of intravenous infusions of ACTH in the form of relatively crude preparations of porcine origin into two young male medical students and of the rate of disappearance of the ACTH from the circulation Blood was drawn just before beginning the infusion and at several times during and after the infusion The plasma was frozen promptly

and lyophilized in order to guard against loss of activity on standing and was reconstituted for injections at a dose level of 2 ml per hundred grams into the test hypophysectomized rats using the assay method of M A Sayers *et al* (1948) The control plasma and samples collected from 3 to 24 hours after the infusion were negative Based on the laboratory s experience of an MED of 0 2 milliunit in the assay method it was concluded that there was less than 10 milliunits of ACTH per hundred milliliters in normal plasma Obviously therefore a very delicate test had not been conducted During and shortly after the infusions the results were consistent with ACTH concentrations ranging from 50 to 100 milliunits per hundred milliliters indicating that the lyophilization the reconstitution procedure and the biological assay method were not incompatible with the detection *of ACTH (at least of the type administered) in the presence of* human plasma The possibility that an underestimation of the ACTH concentration had occurred was of course not eliminated

Previous reports of attempts to test for ACTH in human plasma or serum were reviewed by Taylor *et al* (1949) and Paris *et al* (1954) In all but one instance (Cooke *et al* 1948) in which the data were presented in inadequate detail for critical evaluation unreliable biological assay methods with intact mice or rats were used and these reports will not be cited again here

Taylor *et al* (1949) tested serum from 3 normal men and 4 normal women for ACTH by the Sayers assay method using 2 or 3 rats for each sample of serum and a dose level of 2 to 3 ml per rat The results were not statistically significant when considered as a group nor were the results from any individual sample of serum such as to suggest that a detectable amount of ACTH was present in any single case Since no standard ACTH was tested in parallel with the sera no information about the lower limit of detectability under the conditions of the test was made available It is probably safe to assume however that the MED was of the order of 0 2 to 0 25 milliunit and *that again as with the first tests by Sayers not a very delicate test had* been performed

After collection of the blood by Taylor *et al* (1949) it was allowed to clot presumably at room temperature before centrifugation The sera were stored in the refrigerator before injection for varying periods of time up to 24 hours and there is a possibility that ACTH was unstable under these conditions

A more delicate test for the presence of ACTH in human blood

was conducted by Sydnor *et al* (1953b) The Oxycel technique was used for concentrating the ACTH in the blood of 7 normal male subjects so that the equivalent of 25 to 50 ml of blood (not plasma) could be injected into each test rat

Again the results were judged to be negative although in 3 cases the reduction in adrenal ascorbic acid averaged 31 42 and 34 mg per hundred grams values regarded by some investigators (Taylor *et al*, 1949 for example) as positive Similar negative results were reported by Hajdu and Forgacs (1954) Sydnor *et al* (1953a) also tested Oxycel extracts of the blood of 9 children age 3 to 13 years injecting the equivalent of 20 to 40 ml of blood into each test rat (2 to 3 per sample) All tests were negative for ACTH

Still additional support for the idea that the ACTH concentration of normal human plasma is low came from Bethune *et al* (1957) who failed to detect any ACTH in the plasma of 8 normal human subjects and 8 hospitalized patients tested by the method of Nelson and Hume (1955) In each case 20 to 30 ml of unfractionated plasma was administered to the test hypophysectomized dog Since the MED of ACTH in this method is 0 75 to 1 5 milliunits the negative results indicated that there was less than 2 5 to 7 5 milliunits of ACTH per hundred milliliters of plasma

Quantitative Estimates Low

Fujita (1957) was the first of the group of investigators whose results indicate a low concentration of ACTH in normal blood to carry the analysis far enough to derive quantitative information from his data He concentrated the ACTH in the blood by a modified Oxycel procedure so that the equivalent of 200 ml of blood could be administered intravenously to each test hypophysectomized rat The blood from 27 normal subjects was tested at this single dose level in one to two test rats each in comparison with a low but effective dose (0 25 milliunit) of standard ACTH Blood from 6 of the subjects was tested twice for a total of 33 determinations In approximately half the tests the adrenal ascorbic acid response was greater than that to 0 25 milliunit of ACTH Fujita concluded that the mean concentration of ACTH in the blood for all the subjects was approximately 0 1 milliunit per hundred milliliters a result entirely consistent with the results of all previous investigators who were unable to detect ACTH at the dose levels tested

Concentration of the ACTH in normal plasma by a different type

of method (methods 6 and 9 of Cohn *et al* 1946 and Oncley *et al* 1949) by Bethune *et al* (1958) and testing by the bioassay method of Nelson and Hume (1955) also provided a low estimate although somewhat higher than that of Fujita (1957) Four pools of plasma were tested and ACTH activity was invariably found in Fraction II + III The equivalent of 100 to 300 ml of plasma was injected into the test dog An additional amount of ACTH was found in either Fraction IV 1 or IV 4 in the case of three of the four pools Based on the amount found in Fraction II + III alone the average for the three pools in which this fraction was tested was 0 5 milliunit per hundred milliliters of plasma An additional 0 5 milliunit per hundred milliliters on the average was found in Fractions IV 1 and IV 4 for a total of 1 0 milliunit per hundred milliliters subject because of the limited number of tests to a considerable error of estimation This value is five times that reported by Fujita assuming that all the ACTH of blood is in the plasma The discrepancy between the two estimates may be due to incomplete recovery of ACTH from blood by Fujita s Oxycel procedure or as suggested by Nelson (personal communication) the pituitary glands of blood donors for Bethune *et al* (1958) may have been stimulated to secrete ACTH at an increased rate by the blood collection procedure The distribution of the ACTH from normal plasma in the various Cohn fractions was in general confirmed by similar experiments in which standard ACTH was added to week-old plasma before fractionation or in which plasma from patients receiving continuous intravenous infusions of ACTH was fractionated

Quantitative Estimates High

Thus far only the data from investigators who failed to detect any ACTH in normal human plasma or who found very small concentrations 0 1 to 1 0 milliunit per hundred milliliters have been reviewed Certain other investigators have claimed to be able to detect ACTH in normal plasma even when it is injected into test animals in small doses and have concluded that the normal ACTH concentration of plasma is 100 milliunits or more per 100 ml

The first of such reports was by Bornstein and Trewhella (1950) Based on acid acetone extracts of the plasma of 12 normal subjects age 17 to 61 years and the assay method of M A Sayers *et al* (1948) a mean concentration of 197 ± 39 µg of ACTH per 100 ml was estimated (La 1 A Munson *et al* 1950 had not yet been adopted as the

international standard but the preparation used by Bornstein and Trewhella appears to have been of approximately equivalent potency so micrograms may justifiably be translated directly into milliunits) The actual data given for the loss of adrenal ascorbic acid following injection of the plasma extract were for the most part between 80 and 120 mg per hundred grams of adrenal weight values that are usually regarded as unequivocal evidence for ACTH On the face of it therefore the data presented by Bornstein and Trewhella in support of their claim to have detected relatively high levels of ACTH in normal human plasma appeared to be adequate

In a later paper Bornstein *et al* (1952) attempted to substantiate the earlier results with a different biological assay method that of Simpson *et al* (1943) in which rats are given an injection twice daily for 14 days beginning immediately after hypophysectomy At the end of the assay the adrenal glands are weighed and evaluated histologically in comparison with those from control hypophysectomized rats The MED for this assay method is much higher than that for the adrenal ascorbic acid method 20 to 80 milliunits per day as opposed to 0 2 to 0 25 milliunit for a single injection The results after the twice daily injection of extract equivalent to 5 ml of plasma were only slightly different from the controls although in one of the two experiments the difference was statistically significant At autopsy all test rats were examined for completeness of hypophysectomy and the data from the rather high proportion of rats found to be incompletely hypophysectomized were discarded This valuable control procedure was not mentioned in the paper by Bornstein and Trewhella (1950) and one wonders if the positive results for ACTH reported there may not have been due to incomplete hypophysectomy of the test rats

In an extension of the work of Bornstein *et al* (1952) a clear confirmation was not obtained by Gray and Parrott (1953) Acid acetone extracts of normal human plasma showed no significant effect on adrenal ascorbic acid in hypophysectomized rats However in a study designed to improve the efficiency of the acid acetone fractionation procedure it was observed that in some instances the adrenal ascorbic acid response to standard ACTH added to plasma or plasma extract was greater than to standard ACTH alone This was the case for 3 out of 6 normal female subjects tested For these 3 cases a plasma ACTH concentration of 60 to 91 milliunits per

hundred milliliters was estimated but the statistical significance of the results may be questioned

A second group of investigators (Montanari *et al*) at the University of Bologna has also reported rather high concentrations of ACTH in the plasma of normal human subjects A careful study of their reports has not revealed any obvious error in technique or interpretation and the data and conclusions remain as unexplained contradictions to the work of most other authors

In the first reports of the Bologna group (Montanari *et al* 1951 1953) the plasma of 8 normal men was tested by the method of M A Sayers *et al* (1948) The dose of plasma administered to each hypophysectomized rat was 0 5 ml per hundred grams of body weight and 2 to 3 test rats were used for each sample The mean decrease in adrenal ascorbic acid for each sample of plasma varied from 37 to 67 mg per hundred grams with an over all mean of 55 ± 3 5 The ACTH potency was estimated from a standard curve obtained with an ACTH preparation contributed by C H Li which may be assumed to be equivalent in potency to La 1 A The mean value for 0 5 ml of the 8 normal plasmas corresponded to 0 8 μg (0 8 milliunit) of Li s ACTH and multiplying by the appropriate factor of 200 yielded as the estimate for normal plasma ACTH 160 μg (milliunits) per hundred milliliters not unlike that originally stated by Bornstein and Trewhella (197 ± 39)

Special pains were taken by Montanari et al (1951 1953) to guard against instability of the ACTH The blood was centrifuged at 4°C immediately after collection and the plasma was injected into the test animal immediately thereafter The authors concluded from the data that after 4 hours standing at 18 to 20 C 49 per cent of the ACTH activity had been lost and after 12 hours almost all the activity had disappeared They also stated that plasma fractionated with acid acetone according to the procedure of Bornstein and Trewhella (1950) assayed only 39 per cent as much ACTH as when the plasma was injected directly On the other hand the activity was found to be ultrafiltrable through cellophane at 15 atmospheres pressure (Rossi *et al* 1952 1953) Only 30 minutes was required for completion of the procedure which was conducted at 2 C The mean reduction in adrenal ascorbic acid following administration of 0 5 ml of ultra filtrate per hundred grams to each test rat was 47 ± 4 2 mg as compared with 36 ± 3 6 mg per hundred grams after injection of the

original plasma It would appear from these data that the ACTH activity found in normal plasma by the Bologna group is stable for at least 30 minutes

Subsequently Moruzzi *et al* (1954) tested plasma from 6 additional normal subjects and obtained similar results Using doses of 0 5 ml of plasma per rat the mean reduction in adrenal ascorbic acid (groups of 3 to 4 rats) varied from 19 to 87 by reference to the standard curve with La I A equivalent to 30 to 144 milliunits per hundred milliliters of plasma In two additional papers (Moruzzi *et al* 1953 Martinelli and Montanari 1955a) the average estimated plasma ACTH levels reported for 5 male subjects 20 to 45 years old and 5 male subjects 20 to 40 years old were respectively 47 and 35 milliunits per hundred milliliters When the same plasma was subjected to acetic acid extraction and adsorption and elution from Oxycel no ACTH effect could be detected

On the basis of these and certain other more remote observations the Bologna group came to the conclusion (Moruzzi *et al* 1953 1954) that there are two types of ACTH in human plasma active as found by them by the direct injection of untreated plasma and activatable ACTH which becomes active when plasma is processed through the Oxycel extraction procedure In contrast to the values cited above for healthy young men Martinelli and Montanari (1955a) failed to detect any active ACTH in the plasma of 5 elderly men aged 65 to 75 years but activatable ACTH was estimated at 33 milliunits per hundred milliliters No activatable ACTH was found in the plasma of the young men In a more detailed presentation of tests on 10 elderly men aged 65 to 90 and 5 young men aged 25 to 42 Martinelli and Montanari (1955b) reported similar results active ACTH in the plasma of the young men but no activatable ACTH activatable ACTH in the plasma of the old men but no active ACTH However the data seem rather variable for definite conclusions with mean adrenal ascorbic acid values ranging for example in the tests for active ACTH in old men s plasma from a gain of 12 mg to a loss of 35 mg per hundred grams and in the tests for activatable ACTH in young men s plasma from a gain of 75 mg to a loss of 2 mg per hundred grams

Relatively high normal plasma ACTH concentrations were also reported from two other laboratories Ceresa and Reyneri (1953) tested acid acetone extracts of normal plasma by the Sayers method The results expressed as mean reduction of adrenal ascorbic acid

following administration of extract equivalent to 1 ml of plasma ranged from 81 to 98 mg per hundred grams and were stated to correspond to 108 to 173 μg (milliunits) of ACTH per hundred milliliters Gaarenstrom *et al* (1954) tested 33 female patients (presumably normal subjects) for plasma ACTH The acid acetone extract of 20 ml plasma was administered to each test rat (the Sayers method) using only one rat per subject The resulting change in adrenal ascorbic acid ranged from +80 to −130 mg per hundred grams Assuming that a fall of 25 mg denoted a significant effect ACTH was detected in 16 of the 33 samples tested but the authors conceded that this was a dubious conclusion considering the total variability of the results The vulnerability of conclusions about plasma ACTH based on data that are scanty or of questionable significance is well exemplified by this study of Gaarenstrom *et al*

Critical Attack on High Estimates of ACTH Levels in Normal Human Plasma

The problem of the discrepant estimates of ACTH concentration has been attacked editorially by G Sayers (1955) An attempt was made to explain the relatively high ACTH concentrations reported by Bornstein Montanari and their collaborators either on the basis of possible technical errors or by pharmacological reasoning The two principal sources of technical error suggested were (1) incomplete hypophysectomy of the test rats which would allow the aspects of stress of the assay procedure to stimulate secretion of ACTH from residual pituitary tissue and therefore record a falsely positive response and (2) contamination of the plasma with ACTH Although these considerations are important in all ACTH assays they are not the obvious answer to Bornstein and Trewhella (1950) who obtained a low value in a case of Simmonds disease (hypophyseal cachexia) or to Moruzzi *et al* (1954) who also reported plasma samples without detectable ACTH On the pharmacological side Sayers pointed out the obvious inconsistency of normal blood levels of 200 milliunits of ACTH per hundred milliliters with the absence of adrenal cortical hyperactivity whereas calculations from the rate of intravenous infusion known to be sufficient for maximal stimulation of the adrenal cortex and the rate of disappearance of ACTH from the circulation show that plasma levels even as high as 20 milliunits per hundred milliliters would be associated not with normal function but with

hyperactivity of the adrenal cortex and the attendant clinical manifestations

It may safely be concluded that present available data are not adequate for a definitive statement of the normal or average concentration of ACTH in human plasma A number of investigators have concluded that it is relatively low and there are data to show that it is from 0 2 to 1 milliunit per hundred milliliters on the average

However the possibility that this range is an underestimation due to instability of the ACTH under the conditions of storage or the method of concentration employed cannot be excluded Although this reviewer tends to favor a low estimate of plasma ACTH under normal circumstances it must be conceded that there are no published data in which the exact conditions of rapid collection and injection of plasma described by the Bologna group have been duplicated to contradict directly the early claim of this group (Montanari *et al* 1951 1953) that the true normal value is higher than 100 milliunits per hundred milliliters The different values reported from year to year by the Bologna group for normal plasma ACTH have tended to become lower and lower 160 milliunits in 1951 and 1953 (Montanari *et al*) 47 milliunits in 1953 (Moruzzi *et al*) 19 to 87 milliunits in 1954 (Moruzzi *et al*) and 35 milliunits per hundred milliliters in 1955 (Martinelli and Montanari) The last value given 35 milliunits per hundred milliliters of plasma corresponds to a fall in adrenal ascorbic acid of only 25 mg per hundred grams (Moruzzi *et al* 1954) a dangerously low result in the reviewer s opinion even for presumptive identification of ACTH No publications on plasma ACTH from the Bologna group have appeared since 1956 and unless further confirmatory work is forthcoming the conclusions of this group and others who have reported relatively high values for normal plasma ACTH are likely to recede gradually into oblivion

PLASMA ACTH LEVELS IN DISEASE

ACTH Levels in Addison s Disease

The first presumptive evidence for the presence of an increased concentration of ACTH in the serum (or plasma) of patients with Addison s disease was published by Taylor *et al* (1949) In contrast to uniformly negative results when sera from normal subjects or patients with adrenal cortical hyperfunction were tested for ACTH by the method of M A Sayers *et al* (1948) the administration of

2 5 to 3 0 ml of serum from 5 untreated patients with Addison s disease was followed by a statistically significant ($P < 01$) decrease in adrenal ascorbic acid of the recipient hypophysectomized rats The mean decline in adrenal ascorbic acid was only 22 ± 5 mg per hundred grams however which would be considered by other workers to be a rather low value for an unequivocal conclusion Serum from jugular vein blood from 2 patients gave somewhat higher results suggesting that blood from the head may contain more ACTH than blood from an extremity In a later paper from the same laboratory (Paris *et al* 1954) 2 additional patients with Addison s disease were reported The mean loss of adrenal ascorbic acid in the test rats was 38 and 48 mg per hundred grams respectively after administration of 2 5 ml of serum

The results of these studies indicated only rather tenuously that the ACTH concentration of human blood is increased in adrenal insufficiency Because no reference standard was used the ACTH concentration could not be estimated quantitatively In fact the authors stated that with standard ACTH they were unable to obtain consistently significant regressions of response on log dose

Sydnor *et al* (1953b) assayed blood (after concentration by the Oxycel technique) from 3 patients with Addison s disease The equivalent of 25 ml of blood was administered to each test rat according to the method of M A Sayers *et al* (1948) The mean results in terms of milligrams of ascorbic acid lost per hundred grams of adrenal weight were 60 81 and 215 respectively with the corresponding mean effects of 25 milliunits of standard ACTH of 55 and 45 Again a quantitative bioassay was not attempted but roughly speaking values ranging from 1 to 10 milliunits per hundred milliliters of blood might be estimated

A more extensive study of the ACTH concentration of plasma in Addison s disease was made by Bethune *et al* (1957) using the assay method of Nelson and Hume (1955) (adrenal venous steroids in the hypophysectomized dog) Tests were conducted on 32 such patients, among whom there were 3 who had been bilaterally adrenalectomized The dose of plasma injected into the test dog was 20 to 30 ml and the MED for ACTH in this method being approximately 0 75 to 1 5 milliunits the minimum detectable concentration was from 2 5 to 7 5 milliunits per hundred milliliters Although the amount of plasma that could be made available from each patient at any one time was insufficient to permit testing each sample more than once some of the

individual patients were tested at several different times It was possible to detect ACTH in the plasma of 26 of the 32 patients on at least one occasion It was not possible to detect ACTH in the plasma of 2 of the patients at any time even though tested on 2 to 4 different occasions There were 4 others who were tested only once and in which results were negative Three additional patients tested negatively on one of several occasions The positive tests showed ACTH concentrations ranging from 2 6 to 30 0 milliunits of ACTH per hundred milliliters with a mean of 8 4 ± 7 4 as compared with no detectable ACTH activity in the plasma of 8 normal subjects and 8 hospitalized patients

Extremely high levels of plasma ACTH (150 to 400 milliunits per hundred milliliters) were found by Nelson *et al* (1958) in a female patient who had been bilaterally adrenalectomized for Cushing s syndrome 3 years previously In this patient an ACTH producing chromophobe tumor of the pituitary gland was identified and removed surgically Three tests for plasma ACTH in the 2 weeks immediately postoperative were negative but at 3 weeks after the operation the plasma ACTH was estimated as 20 milliunits per hundred milliliters and in five additional assays during the following 3 months the plasma ACTH varied between 8 and 14 milliunits per hundred milliliters values within the normal range in Addison s disease Residual pituitary tissue that eluded the surgical hypophysectomy was undoubtedly responsible for the resumption of ACTH secretion

Effect of Adrenal Cortical Steroids on Plasma ACTH The inhibitory effect of adrenal cortical steroids on the secretion of ACTH is a well established fact abundantly substantiated by a variety of experimental observations in man and laboratory animals For the most part the data were obtained by indirect measurements Bethune *et al* (1957) however determined the plasma ACTH directly by the method of Nelson and Hume (1955) after intravenous infusions of small to moderate doses of cortisol in 5 patients with Addison s disease who had measurable concentrations of ACTH in the plasma at the beginning of the infusion A conclusive decline to undetectable levels of ACTH occurred after 8 hours (the duration of the infusion) in the 3 patients infused with a total of 20 mg of cortisol In one of these patients who was also tested at 2 hours the fall in plasma ACTH had already occurred after the infusion of only 5 mg of cortisol In the other tests (there were 9 tests in all) lower total doses of cortisol 2 5 to 10 mg were infused and in only one of the tests

did the plasma ACTH fall to an undetectable level before or by the end of the infusion In at least 3 of the tests with lower doses there was no significant change in plasma ACTH at the times of observation It may properly be concluded therefore that an adequately large dose of cortisol can reduce the level of plasma ACTH in Addison s disease within a few hours presumably by inhibiting the rate of secretion by the anterior pituitary gland The failure of lower doses to reduce plasma ACTH consistently within a standard 8 hour time interval exemplifies the quantitative dose related nature of the inhib itory action of the cortical steroids on the pituitary gland The fact that 10 mg in 8 hours was not a consistently inhibitory dose of cortisol although it is not greatly different from the presumed normal rate of secretion leaves open the possibility that a longer duration of administration of cortisol at the same rate would have reduced the plasma ACTH to a normal level

The less extensive results of earlier investigators are generally but not entirely in accord with the work of Bethune *et al* (1957) Taylor *et al* (1949) and Paris *et al* (1954) reported that the plasma of 3 pa tients with Addison s disease receiving clinically adequate replacement therapy did not contain a detectable concentration of ACTH in contrast to the results with untreated Addisonians (see p 175)

The results of Sydnor *et al* (1953a b) on the effect of cortical steroids were rather variable In 3 presumably adequately treated patients with Addison s disease ACTH was still detected in the blood and at a level not dissimilar from that observed for 3 untreated patients On the other hand during cortisone therapy of 5 children with congenital adrenal hyperplasia ACTH was not detectable in the blood although before treatment (see p 178) the tests for ACTH in the same children had been positive The blood of 4 of the children was again assayed for ACTH after cessation of cortisone administra tion but an increased blood ACTH was detected in only 1 child (Sydnor *et al* 1953a Kelley *et al* 1953) In these studies the biological assay method was that of M A Sayers *et al* (1948) and Oxycel extract equivalent to 20 to 40 ml of blood was administered to each test rat

Congenital Adrenal Hyperplasia with Virilism

The basis of congenital adrenal hyperplasia with virilism is almost certainly a deficiency of adrenal cortical hydroxylating enzymes im portant in the biosynthesis of cortisol (Wilkins 1957) The resulting

low level of circulating cortisol relieves the anterior pituitary gland from the normal inhibition permitting an increased rate of secretion of ACTH which in turn stimulates the adrenal cortex to synthesize and secrete the androgenic steroids for which it has adequate mechanisms in abnormally increased amounts

Sydnor *et al* (1953a) tested the blood of 5 children with congenital adrenal hyperplasia for ACTH using the Oxycel technique doses equivalent to 20 to 40 milliliters of blood and the bioassay method of M A Sayers *et al* (1948) The mean fall in adrenal ascorbic acid in the test rats was 54 ± 8 mg per hundred grams probably a meaningful as well as statistically significant value A detectable amount of ACTH was not found in the blood of control children in the same study One additional patient with blood positive for ACTH was added to the series by Kelley *et al* (1953)

Paris *et al* (1954) tested 3 patients ACTH was detected in the plasma of only one of them However the test dose was only 2 to 2 5 ml of untreated plasma much less than that used by Sydnor *et al* (1953a) Four patients with adipose gynism were studied by Gray and Parrott (1953) by their method (reviewed adversely in an earlier section) it was concluded that ACTH was detected in the plasma of 3 of them

In the series of Bierich *et al* (1955) blood from 4 patients was tested Oxycel fractionation and the assay method of M A Sayers *et al* (1948) were used with doses equivalent to 25 ml of blood The mean decrease in adrenal ascorbic acid 9 to 15 mg per hundred grams although possibly statistically significant would be regarded by most workers as too low to be good evidence for ACTH indeed the authors concluded only that the results were suggestive of a possible increase in ACTH

The weight of evidence suggests that plasma ACTH is elevated in congenital virilizing adrenal hyperplasia but to a very modest extent so much so that it has been possible barely to detect the presence of ACTH let alone quantitate it and that not consistently in the hands of all investigators

Cushing s Syndrome

The Mayo Clinic group (Taylor *et al* 1949 Paris *et al* 1954) tested the plasma of 7 patients with active Cushing s syndrome for ACTH The bioassay method of M A Sayers *et al* (1948) was used and 2 to 3 ml of plasma was injected into each test rat Therefore

assuming an MED of 0 2 milliunit concentrations of less than 5 to 10 milliunits per hundred milliliters could not be detected No ACTH was detected in plasma from 4 of the 7 patients In the remaining 3 patients one with adrenal hyperplasia (27 mg) one with an adrenal tumor (29 mg) and one with acromegaly (60 mg) a detectable concentration of ACTH was found according to the authors criterion which was that a mean loss of adrenal ascorbic acid of at least 20 mg per hundred grams must have occurred in the test rats The numbers in parentheses denote the mean loss of adrenal ascorbic acid in each case and show that certainly in 2 of the 3 patients the results were of no more than borderline significance The fact that these values for Cushing s syndrome are not greatly different from the values found by the same authors in Addison s disease a condition in which an elevated plasma ACTH level has been rather well substantiated does not strengthen their significance The patient with an adrenal tumor was retested after removal of the tumor with no significant change in the result Tests for plasma ACTH in 2 additional patients with Cushing s syndrome after removal of an adrenal tumor were negative They had not been tested previously

Sydnor *et al* (1953b) tested the blood of 3 patients with Cushing s syndrome for ACTH The bioassay method of M A Sayers *et al* (1948) was used and Oxycel extracts of 22 to 25 ml of blood were injected into each test rat The results were compared with controls and the effect of 0 25 milliunit of standard ACTH In none of the 3 patients could ACTH be detected with certainty although in 1 patient the mean loss of 22 mg in adrenal ascorbic acid would have been regarded as positive by Taylor *et al* (1949) A negative result in this study by Sydnor *et al* (1953b) implies that the ACTH concentration was less than 1 to 2 milliunits per hundred milliliters of blood

The plasma of patients with Cushing s syndrome was also assayed for ACTH by the previously cited investigators who have reported relatively high values for plasma ACTH in normal human subjects In all cases values higher than normal were reported Data were obtained from 3 patients by Bornstein and Trewhella (1950) from 4 patients by Gray and Parrott (1953) and from 1 patient by Martinelli *et al* (1954) The mean plasma ACTH was estimated by Bornstein and Trewhella to be 451 ± 105 μg (equivalent to milliunits) per hundred milliliters as compared to 197 ± 39 μg for 12 normal subjects Gray and Parrott reported a range of 91 to 305 μg considerably above that for normal subjects Martinelli *et al* also found plasma

ACTH to be elevated in their patient with Cushing's syndrome Both the active and activatable ACTH fractions as defined by these authors were elevated

It should not be surprising to find a wide variation in plasma ACTH from one case of Cushing's syndrome to another depending on whether the fundamental abnormality rests with the adrenal cortex the anterior pituitary gland the hypothalamus or a combination of sites In conjunction with other studies reliable estimations of plasma ACTH may in the future be expected to contribute importantly to a clarification of the pathophysiology of Cushing's syndrome The currently available data however are not very informative In the reviewer's opinion the results of Taylor *et al* (1949) Paris *et al* (1954) and Sydnor *et al* (1953a b) may all safely be regarded as negative for ACTH at the doses tested The positive results of the other investigators are subject to the same reservations already stated (p 173)

Other Endocrine Abnormalities

Acromegaly One case of acromegaly coexistent with Cushing's syndrome with detectable plasma ACTH was cited in the previous section (p 179) Paris *et al* (1954) reported on 2 other patients with acromegaly who had detectable concentrations of ACTH in plasma One of these patients was subsequently hypophysectomized with a concomitant fall in plasma ACTH One patient with mild acromegaly and hirsutism was reported by Gray and Parrott (1953) to have an elevated plasma ACTH

These uniformly positive reports of elevated plasma ACTH in acromegaly would not have been anticipated by the reviewer and additional tests of patients with acromegaly will be awaited with interest

Pheochromocytoma Two patients were studied by Paris *et al* (1954) One patient was studied before and at three intervals after surgery The other patient with the paroxysmal variety was tested for plasma ACTH during an asymptomatic period after a provocative dose of histamine and 9 days postoperative The dose of untreated plasma was 2 ml per rat In three of the seven tests two preoperative and one postoperative the mean fall in adrenal ascorbic acid (25 to 39 mg per hundred grams) in the test rats was judged positive for ACTH by the authors In the reviewer's opinion however the responses of the test rats were so near the normal variation of controls

that it is by no means certain that ACTH was detected in any of the plasma samples

Hypopituitarism One patient with acromegaly after hypophysectomy and 1 patient with panhypopituitarism were tested for plasma ACTH by Paris *et al* (1954) The tests were negative Bornstein and Trewhella (1950) reported a very low value for plasma ACTH if any at all in 1 female patient with Simmonds disease and a value lower than normal in their series for 1 case of hypopituitarism with dwarfism and obesity Martinelli *et al* (1954) detected no active ACTH in the plasma of their patient with hypopituitarism and a very low level if any of activatable ACTH

Hypothyroidism Paris *et al* (1954) reported tests for plasma ACTH in 6 patients with myxedema The results at a dose level of 2 5 ml of plasma per rat were variable with average losses of adrenal ascorbic acid ranging from 3 to 52 mg per hundred grams Therefore according to the criterion of the authors there was an elevated concentration of ACTH in the plasma of at least 1 of the patients

Hyperthyroidism Montanari *et al* (1956) found a relatively high concentration of active ACTH in the plasma of 2 patients with Basedow s disease (toxic diffuse goiter) but no activatable ACTH was detected Contrariwise in 2 patients with thyrotoxicosis struma retrosternale a high level of activatable ACTH and no active ACTH were reported

In the series from the Mayo Clinic (Paris *et al* 1954) 3 patients with exophthalmic goiter were examined By the assay method of M A Sayers *et al* (1948) the results were reported as variable with a mean fall in adrenal ascorbic acid of 4 to 29 mg per hundred grams *indicating a possibility that ACTH had been detected in the plasma* of 1 of the patients

Diabetes mellitus Bornstein and Trewhella (1950) reported no significant elevation of plasma ACTH in 6 patients with diabetes mellitus The values given were 202 $\pm$ 27 milliunits as compared with 192 $\pm$ 25 milliunits per hundred milliliters for normal subjects Data for diabetic pregnant women are given under Pregnancy below

Plasma ACTH Levels in Miscellaneous Conditions

Bornstein and Trewhella (1950) reported elevated plasma ACTH in 6 patients with uncontrolled *congestive heart failure* (412 $\pm$ 41 milliunits per hundred milliliters) but when the same patients were retested after their condition had been controlled the values for

plasma ACTH fell to the level stated by these authors to be normal (192 ± 25 milliunits as compared with a normal level of 197 ± 39 milliunits)

Bornstein and Trewhella (1950) reported a significant elevation of plasma ACTH after *surgery* (337 ± 25 milliunits per hundred milliliters)

Sydnor *et al* (1953a b) reported detection of ACTH in the blood of 1 patient with *idiopathic hypoglycemosis* and in 2 patients with *acute rheumatic fever with carditis* In one of the latter patients whose blood extract produced a fall of 145 mg per hundred grams in the test rats adrenal ascorbic acid the blood ACTH receded to an undetectable level after therapy Sydnor *et al* failed to find ACTH in the blood of 1 patient with *acute rheumatic fever without carditis* in 1 patient with *acute glomerulonephritis* in 2 patients with *Sydenham s chorea* in 1 patient with *anorexia nervosa* and in 2 patients with *tuberculosis* In these tests by Sydnor *et al* each test rat received Oxycel extract equivalent to 22 to 40 ml of blood

A mean fall in adrenal ascorbic acid of 39 mg per hundred grams was observed by Paris *et al* (1954) following administration of 2 5 ml of plasma from a woman with *dysmenorrhea* possibly indicating an elevated plasma ACTH concentration At a later date when the patient was asymptomatic the test for plasma ACTH was definitely negative

PREGNANCY — ITS COMPLICATIONS LACTATION, AND THE POSTPARTUM

In the studies of Paris *et al* (1954) the change in the mean adrenal ascorbic acid concentration of test rats each given 2 to 2 5 ml of serum or plasma from 16 normal pregnant women ranged from a rise of 18 to a fall of 37 mg per hundred grams suggesting that in pregnancy there is a marked rise in plasma ACTH in only a minority of women if any The tests for plasma ACTH were also negative in 1 case of threatened abortion (on steroid therapy) in 2 lactating women and in 1 woman post partum In only 1 of 2 cases of pre eclampsia was there a possibly positive test for plasma ACTH — a mean fall in adrenal ascorbic acid of 37 mg per hundred grams

Gray and Parrott (1953) reported the presence of ACTH in the plasma of 1 normal pregnant woman but the estimated concentration

was in the same range as that found in nonpregnant women by these authors

The plasma of 13 diabetic pregnant women was also tested In 6 cases no ACTH was detected In the remaining 7 cases detection of ACTH in the plasma was reported and estimated at 39 to 310 milli units per hundred milliliters However in 2 patients on whom re peated tests were conducted the test was negative on at least one occasion Thus the observation of an elevated plasma ACTH was not an entirely consistent one

Granirer an advocate of the treatment of patients with rheumatoid arthritis with infusions of plasma collected from women within 24 hours post partum tested such plasma for ACTH by the method of M A Sayers *et al* (1948) The fresh plasma was tested at 3 dose levels 0 5 1 0 and 2 0 ml in comparison with 3 dose levels of standard ACTH From the results it was concluded that the ACTH concen tration of the plasma was 180 milliunits per hundred milliliters No ACTH could be detected in the plasma of normal women After storage of pooled post partum plasma at 4°C for 5 weeks tests indi cated that the ACTH concentration had fallen to 56 milliunits per hundred milliliters and after 8 to 12 weeks of storage no ACTH was detected in the plasma These observations (Granirer 1951) have not yet been confirmed

ADRENAL WEIGHT–MAINTAINING ACTIVITY OF PLASMA

Jailer *et al* (1957) discovered that repeated injections of plasma from certain categories of patients into rats beginning immediately after hypophysectomy significantly retarded the loss in adrenal weight The rats were injected subcutaneously with 0 3 ml of plasma three times daily for 9 days

This adrenal weight maintaining activity of plasma was detected in 7 of 9 patients with Cushing s syndrome due to bilateral adrenal hyperplasia in 2 patients with active acromegaly and in women in the third trimester of pregnancy (3 pools of pregnancy plasma were tested) No such activity was detected in the plasma of normal sub jects of 3 patients with Cushing s syndrome due to adrenal tumor or in one plasma pool from patients in adrenal insufficiency

The presence of the adrenal weight factor in plasma from pregnant women was confirmed by Lanman and Dinerstein (1959) who also presented rather impressive data to indicate that the factor responsible

for the effect is chorionic gonadotropin which stimulates secretion of androgen by the hypophysectomized rat s testis The androgen in turn partially prevents atrophy of the adrenal cortex after hypophy sectomy as is known to be the case in rats (Zizine *et al* 1950)

The observations of Lanman and Dinerstein do not explain the effect of plasma from patients with Cushing s syndrome and from patients with acromegaly It was noted by Jailer *et al* that the factor responsible for the effect need not be the same for the three categories of plasma and growth hormone was considered as one possibility in acromegaly

The plasma of 2 patients of Gemzell *et al* (1955) one with acro megaly and the other with gigantism was tested for growth hormone in hypophysectomized rats by the tibia test The responses although statistically significant were rather small probably indicating that the growth hormone concentration in the plasma of these patients at least would be too low to have an effect on adrenal weight Identi fication or further characterization of the factor or factors (other than chorionic gonadotropin) discovered by Jailer *et al* (1957) if it or they are new must await additional investigation

CONCLUSIONS

Biological assays of ACTH in human plasma in health and disease and under various experimental conditions will in the future un doubtedly contribute importantly to a better understanding of the normal and pathological physiology of the anterior hypophyseal adrenal cortical axis The technological basis for reliable ACTH assays in plasma is as yet however still in an early stage of development Consequently at present there is only a rather limited amount of sound information available about human plasma ACTH

Almost all the published data were obtained with two biological assay methods both of them apparently specific for ACTH and under favorable circumstances reasonably precise They are the method of M A Sayers *et al* (1948) in which the fall in adrenal ascorbic acid is measured after the intravenous administration of ACTH to hypophysectomized rats and the method of Nelson and Hume (1955) in which the increase in adrenal venous 17 hydroxy corticoids is determined after intravenous injection of ACTH into the hypophysectomized dog In the application of these two generally excellent methods to blood or plasma however it has not usually

been possible to work under ideal assay conditions In particular it has been rare to assay blood or plasma (directly or as extracts) at two or more dose levels and thus the classic tests of linearity and parallelism for identity of the active principle in standard and unknown preparations have not been available The low concentration of ACTH in plasma on the one hand and the relatively high dose requirements of the assay methods on the other are responsible for this fault

Concentration of the ACTH in blood or plasma in order to permit the effective administration of higher doses has been attempted with varying degrees of success Three methods have been used (1) extraction and precipitation with acid acetone (2) adsorption on and elution from Oxycel and (3) the Cohn fractionation with ethanol Even assuming a reasonably satisfactory method of concentration of the ACTH the problem of obtaining sufficient blood to satisfy the dose requirement of the assay remains a serious one and an assay method that demands less ACTH is needed Two new more delicate assay methods still in the process of development may eventually meet this specification They depend on measurement of the increase in respectively ascorbic acid and corticosterone in the adrenal venous blood of hypophysectomized rats

Using the older well-established methods and injecting the largest dose of untreated plasma that could be tolerated by the test animal or even a considerably higher dose in the form of a concentrated extract several careful and experienced investigators were unable to detect any significant effect with normal plasma or blood However by the use of special techniques that permitted the injection of the equivalent of 100 to 300 ml of blood or plasma two rather discrepant estimates of approximately 0 1 milliunits per hundred milliliters of blood and 1 milliunit per hundred milliliters of plasma have been reported Other investigators have concluded that normal plasma contains more than 100 milliunits of ACTH per hundred milliliters These varying results were reviewed and it was tentatively concluded that the low estimates for normal plasma ACTH are probably more nearly correct although at present no definitive value or range of values is available

In Addison s disease and after bilateral adrenalectomy there is excellent and essentially indisputable evidence that plasma ACTH rises markedly in most patients The average value in a large series of tests was 8 milliunits per hundred milliliters of plasma but the

range of values was large Adequate replacement therapy with cortical steroids has generally been found to reduce plasma ACTH toward the normal level In congenital adrenal hyperplasia with virilism and manifest or assumed adrenal insufficiency also an increase in plasma ACTH has been reported In Cushing s syndrome the published data are variable but in most patients no increase in plasma ACTH was detected Tests for blood or plasma ACTH have been conducted on limited numbers of other patients with a variety of endocrine and nonendocrine disorders but because the results were too limited in extent or were inconclusive for other reasons meaningful interpretation is difficult or impossible and must await additional data

In conclusion it is apparent that there are important technical problems yet to be solved before the full potentiality of assays for ACTH in human plasma as diagnostic and investigative tools in endocrinology can be realized

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CHAPTER VII

Pituitary Gonadotropins

Janet W McArthur

CHEMICAL AND BIOLOGICAL PROPERTIES
METHODS OF ASSAY
DISTRIBUTION METABOLISM AND EXCRETION
LEVELS IN PHYSIOLOGICAL AND PATHOLOGICAL STATES
BIBLIOGRAPHY

The dependence of the gonads upon a blood borne substance (or substances) for the later stages of their development and for their maintenance was established by the classic researches of P E Smith and his students (Smith and Engle 1927 Smith 1930) and of Zondek and Aschheim (1927) These investigators succeeded in inducing precocious sexual maturation in immature rats and mice and in restoring sexual function in hypophysectomized rats by the implantation of whole pituitary glands and of anterior lobe tissue Fractions which produced two different kinds of gonadotropic effects were subsequently extracted from pituitary glands by Fevold *et al* (1931) One which they called follicle stimulating hormone (FSH) produced follicular growth in the ovary the other luteinizing or interstitial-cell stimulating hormone (LH or ICSH) caused luteinization of the follicles which had been stimulated by FSH In the male organism FSH was found to maintain spermatogenesis and ICSH to promote the secretion of androgen by the interstitial cells of Leydig

The discovery of a pituitary gonadotropic principle in the circulation came about accidentally as a consequence of the development of the Aschheim Zondek test for pregnancy Application of this test to all sorts and conditions of amenorrheic women revealed the disconcerting fact that the body fluids of patients whose amenorrhea was ascribable to diminished or absent ovarian function rather than

to pregnancy likewise possessed powerful gonadotropic properties The blood and urine of women experiencing the natural menopause of patients subjected to operative or roentgen castration and of certain nonpregnant women with irregular menses proved capable of stimulating the ovaries of test mice (Fluhmann 1929a b Zondek 1930 1931)

The search for a similar principle in the circulation of nonhuman species has not been prosecuted as vigorously as has the search for the gonadotropins associated with pregnancy However gonadotropic activity has been demonstrated in the blood serum of the gonadectomized thyroidectomized rat (Contopoulos *et al* 1958) in that of the gonadectomized rat (Emery 1932 Hellbaum and Greep 1943 Cozens and Nelson 1958 Contopoulos *et al* 1958) mouse (Martins 1929) and 13 lined ground squirrel (Moore *et al* 1934) and in that of the estrous pig (Lozinski *et al* 1942) and the estrous rabbit (Soliman and Ghanem 1956) and the bred rabbit (McPhail *et al* 1933 Dumont *et al* 1932 1934) Circulating gonadotropins have not been detected during the various phases of the estrous cycle in sheep whether tested for in untreated serum or in concentrates (Bassett *et al* 1955)

CHEMICAL AND BIOLOGICAL PROPERTIES

FSH and LH have now been obtained from hypophyseal tissues in highly purified form and their chemical and biological properties have been subjected to intense scrutiny (see reviews by Li and Evans 1948 Li 1949 Evans and Simpson 1950 Morris 1955 Noble and *Plunkett 1955) Both are glycoproteins* but differ according to the species of origin with respect to their molecular weight isoelectric point chemical composition and immunological properties Notwithstanding the expenditure of considerable effort evidence to indicate that the pituitary gland actually secretes two separate hormones has not yet been adduced The reported detection of two gonadotropic hormones in fractions prepared from pooled plasma (Cohn *et al* 1944) could not be confirmed (McArthur *et al* 1956a) and attempts to isolate separate gonadotropic principles from urinary extracts have so far proved unavailing (Stran and Jones 1954 Johnsen 1955 Rigas *et al* 1958)

The properties of the circulating hypophyseal gonadotropins have as yet received comparatively little attention The plasma of ovariectomized rats produces both ovarian and uterine enlargement in hy

pophysectomized rats although the absence of interstitial cell repair suggests only a follicle stimulating action (Cozens and Nelson 1958) Blood obtained as early as 7 days postoperatively contains detectable FSH activity which increases steadily over a 4 months period

Serum gonadotropins have been studied with the aid of augmentation methods by Evans *et al* (1933) and by Hellbaum and Greep (1943) The latter investigators obtained evidence suggesting that androgen effects the release of LH from the pituitary gland into the circulation and suppresses the liberation of FSH Fevold and Fiske (1939) were unable to detect FSH activity in the serum of rats treated with estrogen However interstitial cell stimulation was produced in intact male rats and when the serum was injected subcutaneously with FSH augmentation and luteinization occurred in female rats These results implied that estrogen had stimulated the release of LH from the pituitary gland

Gonadotropic hormone is present in concentrations too low for detection in the untreated serum of the nonpregnant mare (Cole and Hart 1930) However Cole and Goss (1939) succeeded in demonstrating gonadotropic activity in serum extracts the biological effects of which appeared to be indistinguishable from those elicited by the gonadotropin of pregnant mare s serum Rimington and Rowlands (1944) who applied a somewhat different chemical technique to the serum of the nonpregnant mare were unable to obtain hormonally active extracts

Extracts of the blood of women during menopause produce both follicle stimulation and luteinization in hypophysectomized female and spermatogenesis and interstitial cell repair in hypophysectomized male recipient rats (McArthur *et al* 1956b)

METHODS OF ASSAY

Despite the expenditure of much effort the assay of the hypophyseal gonadotropic principles remains difficult Not only do FSH and LH potentiate one another in many of the biological reactions employed but nonspecific substances contaminating the extracts may exert an augmenting effect (Hamburger 1957) Relatively specific methods for the assay of FSH and of ICSH activity have nevertheless been developed These have been the subject of extensive review (Thayer 1946 Li and Evans 1948 Hamburger 1950 Emmens 1955 Diczfalusy and Heinrichs 1956 Loraine 1956 1958)

Because of the uncertainty concerning the number of gonadotropic hormones the Third International Conference on the Standardization of Hormones (1938) declined to recommend the establishment of an international standard preparation for the measurement of the gonadotropic effects of pituitary preparations or urinary extracts In lieu of an international preparation a number of provisional standards have recently been developed particularly for the assay of urinary extracts (Loraine and Brown 1956 Albert 1956)

DISTRIBUTION METABOLISM AND EXCRETION

The gonadotropic activity of human blood appears to be transported in association with the α_2 and β globulins Bioassay of fractions prepared by Cohn s methods reveals that in both menopausal (McArthur *et al* 1956a) and in nonmenopausal (Antoniades *et al* 1957) plasma the bulk of the gonadotropic activity is concentrated in Fraction II + III and that only traces of activity are detectable in Fractions IV 1 IV 4 and V Somewhat more than half the activity of whole plasma can be recovered in Fraction II + III

The meager information available concerning the metabolism and excretion of circulating pituitary gonadotropin has been summarized by Zondek and Sulman (1945) Like human chorionic gonadotropin and unlike the gonadotropin of pregnant mare s serum pituitary gonadotropin is readily excreted in the urine However the gonadotropins extracted from the urine of adult men and women differ qualitatively from those of the hypophysis (Bahn *et al* 1953a b) Some of the possible reasons for the disparity (differential secretion of FSH and ICSH by the pituitary gland differing rates of metabolism or utilization selective excretion or retention by the kidneys difference in effectiveness of the methods employed for the recovery of FSH and ICSH) have been discussed by Bahn *et al* (1953a) Comparison of the hormonal properties of plasma gonadotropins with those of hypophyseal and urinary origin may be expected to contribute materially to the solution of this problem

Employing a urinary standard (HMG 20_A) for the assay of both blood and urine Apostolakis and Loraine (1960) found the renal clearance of pituitary gonadotropins in 31 postmenopausal women to vary between 0 08 and 0 43 ml /min the mean clearance being 0 21

LEVELS IN PHYSIOLOGICAL AND PATHOLOGICAL STATES

In the years which followed the discovery of gonadotropic activity in the circulation of women with ovarian deficiency Fluhmann and others applied various modifications of the Aschheim Zondek technique to the serum of hundreds of patients The insensitivity of the available assay methods was such as to preclude the recognition of any but elevated gonadotropin levels in untreated serum However the clinical conditions under which such elevated levels might be anticipated were painstakingly defined by Fluhmann (1929a b 1930 1931 1933 1937) by Fluhmann and Murphy (1939) and by Murphy and Fluh mann (1938) An increase of gonadotropic hormone in the blood was found in approximately 40 per cent of patients examined within 3 months after an operative castration and in 76 per cent of those examined after this period in 60 per cent of women following roentgen castration in 60 per cent of postmenopausal women and in a number of patients with protracted amenorrhea Increased gonadotropin levels were sometimes detected as early as 8 days after castration although serial studies were not undertaken The associa tion of elevated serum gonadotropin levels with carcinoma of various pelvic organs reported by Zondek (1931) appears to have been a function of the age of the patients or of gonadal deficiency resulting from tumor growth or from the therapeutic measures employed The gonadotropin content of postmenopausal plasma has been estimated by Apostolakis and Loraine (1960) with the aid of a standard (HMG 20_A) prepared from postmenopausal urine The range in 32 subjects extended from 10 to 55 HMG units per 100 milliliters of plasma the mean being 21

The development of methods for concentrating the gonadotropic activity of serum prior to biological assay enabled additional progress to be made The treatment of serum with organic solvents was found to yield estrogen free precipitates from which the gonadotropic activity could be extracted (Frank 1931 Frank *et al* 1931 Neumann and Peter 1931 1932 Frank and Salmon 1935 Salmon and Frank 1935 Frank and Salmon 1936 Freed 1936 May 1944 Apostolakis 1960) Such extracts of the serum of normal women at first yielded positive responses only when prepared from blood obtained during mid cycle or as in one study (Neumann and Peter 1932) during the late luteal phase However extracts which evoked follicle growth in

the ovaries of recipient rodents were eventually prepared from blood obtained throughout the normal menstrual cycle and luteinization from extracts of blood obtained during mid cycle

Parallel studies have been conducted in the nonpregnant mare in which gonadotropic activity is demonstrable in extracts of serum obtained during all phases of the estrous cycle (Cole and Goss 1939) No qualitative differences were observed in the biological response to extracts representing different phases of the cycle The concentration of hormone appeared to be somewhat greater during metestrus than diestrus although the variation in mares having normal cycles was not marked The highest concentrations of gonadotropin were found in mares with prolonged cycles or in animals which failed to come into estrus during the observation period The occasional false positive tests for pregnancy which have been obtained with unextracted mare s serum (Day and Miller 1940) are generally ascribed to abortion or fetal resorption unknown to owner or attendants However the false positive tests obtained by Varley (1938) in nonpregnant mares with cystic ovaries and by Valle (1947) in 11 13 and 18 year old mares suggest that states of ovarian deficiency in the mare as in women may be associated with elevations of gonadotropin titer

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CHAPTER VIII

*Thyrotropic Hormone(s)**

John F Crigler, Jr

BIOASSAY METHODS

THYROTROPIC HORMONE(S) IN NORMAL HUMAN SERA
- Unfractionated sera
- Fractionated sera

THYROTROPIC HORMONE(S) IN SERA FROM PATIENTS WITH ABNORMAL THYROID STATES
- Thyrotropic hormone(s) in hypothyroidism (congenital spontaneous or induced)
- Thyrotropic hormone(s) in hyperthyroidism (Graves disease)
- Evidence for an abnormal thyrotropic hormone in patients with Graves disease

SUMMARY

BIBLIOGRAPHY

In this chapter the nature and concentration of human serum thyrotropic hormone(s) or thyroid stimulating hormone(s) (TSH) in various physiological states are reviewed and discussed with the presentation of some recently published and unpublished results pertinent to this subject This material is presented more as an aid and a challenge to future investigators than as a compilation of well-established facts since these are not yet available despite a great deal of effort by many workers

While there are excellent reviews on the biochemistry and physiology of pituitary thyrotropic hormone (White 1944 1946 Albert

* The author s work on this problem was begun during the tenure of a fellowship from the National Foundation for Infantile Paralysis and has been continued with support of grants from the National Institute of Arthritis and Metabolic Diseases (A 1295 and A 951) U S Public Health Service and the Smith Kline and French Foundation

1949 Rawson 1949 D Angelo 1954 Hays and Steelman 1955 Sonenberg 1958) information about the content and nature of TSH in human sera has in general been contradictory confusing and difficult to evaluate This has been largely due to limitations of biological assays which for the most part are incapable of measuring the small concentrations of TSH in untreated sera and to problems encountered in attempting to separate thyrotropic activity from other chemical constituents of serum Despite these difficulties recent investigators utilizing improved bioassay methods have begun to obtain results which give a more consistent picture of serum TSH in normal and abnormal thyroid states These findings are increasingly interesting as they support or refute earlier concepts of the role of TSH in regulating thyroid function and as they elucidate the possible chemical nature and properties of TSH in human sera

In the following discussion an attempt has been made to give an accurate comparison of old and new studies of TSH in human sera in terms of the recently established International Unit (Mussett and Perry 1955) * It is hoped that the uninitiated reader as well as future investigators will be assisted by the presentation of the information in this manner however incorrect some estimates may be The material presented is restricted to studies of human sera as other workers (D Angelo 1954 Bottari 1957) have summarized the few quantitative data published on TSH blood concentrations in animals under different experimental conditions

BIOASSAY METHODS

Perhaps nothing has hindered advances in the study of protein hormones — particularly as they exist in biological fluids — so much as the difficulties encountered in detecting their presence by means of biological assay TSH is no exception although the introduction of radioactive isotopes — principally I^{131} — in the study of thyroid function has simplified the measurement of changes in those thyroid parameters which may be reasonably objective and sensitive indices of TSH action

Recent reviews of TSH bioassay methods (Albert 1949 Tala 1952 Lamberg 1953 1955 Wahlberg 1955 Adams and Purves 1957b

The International Unit (I U) was defined as the activity of 13 5 mg of the International Standard for Thyrotropin a pituitary TSH powder blended with lactose to a ratio of 1 19 and made into 20 mg tablets One International Unit is approximately equal to one USP Unit

TABLE I Biological ssays Used in the Stud of TSH in Blood

I Histological Methods

N f m thod	A tho	M thod						Sens ti ty Lm /
		Animal	P ious t tm	M asured po	Inject Typ	N	1 /	
1	Aron (1929 1930 1931 1932)	gui e p g	no	histol g cal		3	4 5	3 0 5 0
2	He tz and Oastle (1936)	t	hyp	hi tological	s c	10	1 2	1 5
3	Starr and Rawson (1938)	gui a pig	none	h sto cell h ight	s c	5	1	2 0
4	D Angelo et l (1942 1951) D Ang l a d Gordo (19 0)	t l tadpole	f t d	hi to c ll h ight	i p	7	0 05	0 025
5	DeRobe ti d Del C te (1944) DeRobe tis (1948)	gui ea pig	no	h t ol loid dr pl t	i	1	2 0	0 02
6	Pu and Grie b h (1949)	gu a pig	thy oxi e	h sto ell h ight		8	~1 0	1 3 2 0
7	U tila nd Kanna (1952)	gu a pig	o e	histo % ep th li m		1	1 0	0 1

II Radioactive Isotopic Methods

N f m thod	Auth	M th d						Sens ti ty l m /
		Animal	Pr tment	Meas ed pons	Injec ns Typ	N	ml /	
8	Que ido t al (1953)	mou	l di ted l	I^{131} ptake	i p	4	1 0	2 5
9	G llil nd and Str dw k (19 3)	chi k	I^{131} thy ine	I^{131} depl tio d a e thy d	s	2	1 5	1 0 2 0
10	Adam and Pu y (1955 1957b)	gu p g	I^{131} thy o l	I^{131} depl t n e in blood	i	1	1 0	0 1
11	G ee pan t al (1956) G ensp nd Lew (1959)	hi k	n	P^{32} ptak	i c	1	0 1	0 25
12	B te d Co f eld (1957)	chi k	I^{131} thy i p pyl th uracil	I^{131} depl ti d c e thyroid		2	1 5	2 0 3 0
13	M Kenzi (1958b)	mo e	I^{131} thy i	I^{131} d pl t n inc s i blood	v	1	0 5	0 05
14	Le y et l (1956) L y and Sol mo (1957)	t	hyp o thy o i	I^{131} ptak	i	1	0 5	0 6
15	C igl (1959)	hick	none	I^{131} ptak	s c	3	1 0	3 0

Abb i tions hypo hypophy ectomy ub taneo i p int ap to eal i int diac i i nou

Lm Int rnational mili unit imated ing th followi g eq i alent lue

1 Int rnati al U t 1USP U i (Mu tt and P ry 1955)
1 USP Unit 10 J nkmann Sch ll Units (Hay and Ste lmann 1955 Bakk t al 1957)
1 J S Unit 1 Heyl Laq U it (Tal 1954 Ov beek t al 1953)
1 Rowlands P rk Unit 2 7 J S Units (Junkmann and Scho ll 1932)

Sonenberg 1958) make unnecessary lengthy discussion of the principles of the procedures that have been described for measuring thyro tropic activity Table I lists the assays which have been used in study ing TSH in blood The table has been divided into two parts Part I

lists assays which utilize the histological response of the thyroid gland as an index of TSH activity The quantitative accuracy of these assays has been increased by improving the method of measuring the histo logical response — for example increase in cell height (Starr and Rawson 1938) or change in per cent epithelium (Uotila and Kinnas 1952) — and by improving the statistical design Nevertheless they remain more tedious and laborious than other types of assays with otherwise comparable attributes (Albert 1949) so that most of the recent studies on TSH have been carried out with the radioactive isotopic methods listed in Part II The responses measured in the latter assays involve alterations in the iodine or phosphorus contain ing chemical constituents of the thyroid gland and therefore permit use of the radioactive isotopes I^{131} and P^{32} to facilitate measurement of the changes induced by TSH In I^{131} assays the initial iodine con tent of the thyroid gland of the test animals is important in deter mining whether thyroidal I^{131} uptake or I^{131} depletion is to be used to measure TSH effect If animals are available with thyroidal I^{131} uptakes greater than 10 to 15 per cent then I^{131} depletion assays measuring either decrease in the I^{131} content of the gland or increase in blood I^{131} are the methods of choice On the other hand if initial thyroidal I^{131} uptakes are less than 5 per cent then assays which measure the increase in I^{131} uptake following TSH administration are preferable

In general the usefulness of a bioassay is determined by its ability to fulfill the ideal attributes — specificity objectivity sensitivity convenience and precision (Albert 1949) — as they apply to the problem under investigation In choosing an assay method for bio logical fluids sensitivity is especially important since concentrations of hormone are usually very low However in the absence of a method for concentrating hormone activity the volume of material that can be injected into each animal may become the limiting factor in deter mining useful sensitivity i e minimum concentration of TSH detectable This problem is best illustrated by considering the stasis tadpole assay of D Angelo *et al* (1942) Sensitivity of this assay is 0 025 International milliunit (I mu) per injection but when expressed in terms of TSH concentration 0 5 I mu per milliliter of TSH is required for detection This concentration of TSH is greater than that required by other bioassay methods which are less sensitive but which utilize animals which can tolerate the injection of larger volumes of test materials (Table I) Therefore in order to interpret reported

observations of serum TSH content it is desirable to know the useful sensitivity of the bioassay under the given experimental conditions In the subsequent tables which report results of studies of TSH in human sera estimates of useful sensitivity have been made and recorded under the heading of minimum TSH detectable (International milliunits per milliliter)

Obviously omitted from Table I are the *in vitro* assays which have recently been reported (Florsheim *et al* 1956 1957 Bakke *et al* 1957 Bottari 1956a b 1957) Except for the method described by Bottari (1957) which measures the effect of TSH on increasing I^{131} uptake in surviving guinea pig thyroid slices these assays have not been used for measuring TSH in biological fluids Perhaps as further experience is obtained with *in vitro* assays they will be more widely used Two of the assays (Bottari 1957 Bakke *et al* 1957) are reported to detect as little as 0 01 I mu per milliliter As methods for isolating TSH from serum are developed these assays may prove especially valuable in studying pituitary thyroid relationships Their sensitivity would permit the measurement of TSH in small amounts of serum and thus allow repeated determinations of serum TSH in a single subject

THYROTROPIC HORMONE(S) IN NORMAL HUMAN SERA

Unfractionated Sera

Table II summarizes the results obtained by various investigators who have assayed unfractionated normal sera for TSH activity Some workers (Aron 1930 1931 1932 1933 Aron *et al* 1932 D Angelo *et al* 1951 Gilliland and Strudwick 1956 DiGeorge *et al* 1957) measured TSH activity but only at the level of minimum TSH detectable by their assay method Other investigators (DeRobertis 1948 Simkin *et al* 1952 Adams 1958) using assays of equal or greater sensitivity found no detectable TSH activity In view of the variability of these results it seems reasonable to conclude that TSH has still not been measured unequivocally in unfractionated normal human sera This conclusion seems particularly justified since no one has reported TSH in unfractionated normal sera determined at two dose levels by increasing the volume injected per animal although the TSH content reported by D Angelo *et al* (1951) and DiGeorge *et al* (1957) would be sufficient to expect a graduated response in several of the other assay techniques Until such a four point assay is obtained the

TABLE II TSH in Unfractionated Normal Human Sera

A tho s	N Sampl s Te ted		Bio ss y		V l p anim l	Min. TSH d tectable	E t TSH m /rang
	Indi	P led	Animal	M asur d e ponse	(ml)	(I.m /ml)	(I.m /ml)
Aron (1930 1931, 1932 1933)	—	—	guinea p g	histo	15	0 6 1 0	detected
Aron et al (1931 1932)							
Hertz and Oastle (1936)	5	—	hypox r t	histo	10 20	0 75	not d tected
DeRob tis (1948)	—	—	gui ea p g	colloid droplet	2	0 01	not detected
Purves a d G ie bach (1949)	4	—	guinea pig	histo	5	1 0	ot detect d
D Angelo et al (1951)	6	3	stasi tadpole	histo	0 35	0 5	0 4 (0 0 8)
Simki et al (1952)	11	—	st sis tadpol	histo	—	—	not d te ted
Asbo Hansen et al (1952)	6	—	stasis tadpole	histo	0 18	1 7	not detect d
Gilliland and Strudw ck (1956)	4	1	chick	I^{131} *deplet on*	5 10	0 1	0 1 (0 0 23)
DiGe rg t al (1957)	3	—	*stasis* tadp l	*histo*	*0 35*	*0 5*	*0 5* (0 0 8)
Adams (1958)	4	—	gui a pig	I^{131} depleti (bl.)	1	0 1	not detected
G een pan a d Lew (1959)	5	—	chick	I^{131} depl tion	2	0 5	t detected

E tim ted ing l ati f as y as publi hed l t (D Georg t l 1957)

question of TSH content of unfractionated normal sera must remain unanswered

Fractionated Sera

Attempts to fractionate human sera in order to concentrate TSH activity have met with varying degrees of success The controversial and often negative results which have been obtained by others using acetone precipitation (Fellinger 1936) cold ethanol fractionation (Cohn *et al* 1946) electrophoretic separation ion exchange chromatography and ethanol saturated sodium chloride extraction of lyophilized plasma (Bates and Condliffe 1959) are summarized in Table III In this table the methods of assay *serum equivalents* injected per animal and the minimum detectable activity per milli liter of serum injected as estimated from the sensitivity of the assays listed in Table I are given when available to assist the reader in

evaluating the significance of the reported results Except for studies carried out by Bates and Condliffe (1959) which have not been confirmed to date all other reports are distinguished by the variability and contradiction in the results obtained by the different workers Cope (1937 1938) and Greenspan and Lew (1959) were

TABLE III TSH in Fractionated Normal Human Sera

A thors	M thod f hemi al fractionati	Bi y		Serum equi pe animal ml	Mi TSH detectabl (I.m /ml)	E t TSH mea /rang (I m /ml)
		A imal	M asured re pons			
Fellinger (1936)	q eous extr t of 85% to e ppt	gui pig	hi to	5 8	—	d t cted
Cope (1937 1938)	aq eou extract of 85% aceto ppt	gui a pig	histo	40 120	0 17	not detect d
Cohn (1945)	C hn f tion IV 4	hick	hist	—	—	detect d
DeRobertis (1948)	q eous extract of 85% tone ppt	guin p g	coll id dr pl t	2 0	0 01	0 05 0 12
Q rido d Lam y (1956)	C hn fractio II III and IV 4	m use	I^{131} ptak	—	—	1 2
P tel (1956)	starch lectro phor sis pH 7 4 Γ/2 0 1	chick	I^{131} ptak	3 0	5 0	t d tected
M Kenzie (1958)	C h f a t o II, III ?	m	I^{131} depleti (bl.)	0 3 1 2	0 08	0 17 (0 1 0 28)
G ee span a d Lew (1959)	aq eou ext act of 85% cet ppt	hick	p^{32} ptake	0 5	0 5	t det t d
	fl w l troph e is lbumin d β γ glob	chi k	p^{32} ptak	0 13 0 17	1 4 1 9	t d tected
Bates a d C nd liff (1959)	EtOH at NaCl extra t lyop plasm	hick	I^{131} d pl ti (thy)	4 8 12	0 19 0 75	0 5
Purves t al (1959)	i hange hr m ac to f ctl	guin a p g	I^{131} d pl t (bl)	200	0 001	not d tected
D Ang lo a d Sunde ma (1959)	C hn f a tio IV-4 III	*sta i tadpol	hi t	—	—	01
	fl w electr phor s s β lb	* tasis tadpole	histo	—	—	0 1

unable to confirm Fellinger s observations on serum fractions prepared by acetone precipitation although DeRobertis (1948) using a highly sensitive but less specific (Dvoskin 1947 1948) assay reported measurable TSH activity Preliminary information reported by Cohn (1946) on the presence of TSH in Fraction IV-4 has been suggestively confirmed by Querido and Lameyer (1956) and by D Angelo and Sunderman (1959) although the TSH content of serum reported

by these two groups differs considerably Both of these groups have found activity at times in other Cohn fractions—Querido and Lameyer (1956) in II + III and D Angelo and Sunderman (1959) questionably in III McKenzie (1958b) has reported finding TSH in II which contains principally the γ globulins D Angelo and Sunderman (1959) have not confirmed McKenzie s results

In relation to this large amounts of both fresh and stored ACD (acid citrate dextrose) and resin collected plasma (see Chapter I) from normal individuals have been fractionated by the methods of Cohn and co workers (Cohn *et al* 1946 Oncley *et al* 1949 Surgenor *et al* 1949 1959) and the resulting fractions studied for TSH activity and TSH inhibition using a chick I^{131} uptake assay (Table I No 15) with a sensitivity of 2 to 3 I mu per injection (Crigler *et al* 1959) In addition concentrated whole plasma and sodium chloride and citric acid eluates of the resin used in collecting the plasma have been assayed The information obtained is summarized in Table IV Al though plasma equivalents of fractions were used in amounts sufficient for TSH to be detected by this assay at concentrations from 0 01 to 0 6 I mu per milliliter of plasma no significant TSH activity was found

Since all injections were 1 ml in volume some of the fractions were highly concentrated protein solutions capable of interfering with the measure of TSH activity either by alteration in absorption or by some specific inhibition as suggested by Postel (1956) and Greenspan and Lew (1959) For this reason TSH standard was added to fractions which were then compared in the same assay to standard TSH at the same concentration in saline and to similar fractions without added TSH Whole plasma concentrated 2 5 times did not alter the re sponse to added TSH Cohn Fractions I and II + III at 4 3 and 5 times plasma concentration respectively significantly ($P < 0\ 05$) sup pressed TSH activity so the fact that endogenous TSH was not detected in these fractions might be explained by the necessity of administering highly concentrated fractions in order to compensate for the limitations of the assay Other Cohn fractions at the concen trations tested did not alter the response to added TSH although the protein concentrations were even greater than in Fractions I and II + III Citric acid eluates of the carboxylic cationic exchange resin IRC 50 used in collecting large volumes of plasma also de pressed the response of the TSH standard This is perhaps best explained by the lability of TSH at pH values below 7 0

Because of the limitations of the chick assay more recent studies have been carried out on the Cohn fractions — particularly Fractions II and IV-4 — using the mouse assay described by McKenzie (1958b) (Table I No 13) Although this assay has a sensitivity of 0 1 I mu per injection in our hands somewhat less than originally reported

TABLE IV Studies of Normal Human Plasma for TSH or TSH Inhibition Using a Chick I^{131} Uptake Assay

Prepar tions	Numbe p ep teated	Plasm Equi alents p anim l ang ml / j	total ml	Min. TSH d tectable† Lm /ml	Est TSH nc Lm /ml	% I^{131} Lptak — Pit TSH 12 5 I m /t j Stand.	Stand plus F act (pl eq / j)
Wh le plasma — c	1	5	15	0 6	ot d te ted	11 9	12 3 (2 5)
PGP‡ — pla ma gl b ppt.	*1*	*23*	*69*	*0 13*	*not d te t d*	—	—
SPPS‡ — stable plasma p tei p natant	1	10	30	0 3	not dete ted	—	—
Fr ti n I (Cohn)§	2	8 6 20	25 8 60	0 15	t d tect d	11 9	8 4 (4 3)
Fr tio II III (Cohn)	3	3 1 10 0	9 3 30	0 3	not detected	11 3 11 9 13 6	11 6 (2 3) 8 3 6 3 (5)
bfr t II, III, III O‖	*4*	*10 20*	*30 60*	*0 15*	*not d te t d*	—	—
Fr t n IV 1 (C h)	4	10 22	30 66	0 14	not det ted	11 4	13 1 (2 5)
Fr tio IV-4 (C h)	6	8 27	24 81	0 11	ot detected	9 4	10 2 (7)
bf t. IV 5 IV 7 9	4	110 200	330 600	0 03 0 01	t d tected	—	—
Fracti V (Cohn)	*3*	*8 13*	*24 39*	*0 23*	*not d tected*	*9 4*	*10 5 (6 6)*
S pe ta t V	2	10 42	30 126	0 07	t d t cted	—	—
IRC 50 1 M N Cl 1 te	3	3 1 25	9 3 75	0 12	t det ted	11 3	9 2 (2 3)
IRC 50 citri acid 1 te	2	3 1 12	9 3 36	0 26	t d te t d	11 3 14 1	7 0 (2 3) 10 9 (2 3)

All animal i ed 3 1 ml injecti t 24 h int rval

† As y d te 3 Lm /injecti th high degree f gnifican

‡ Surgeno et al — 1959

§ Cohn et al — 1946

‖ Oncl y t 1 — 1949

Surg et 1 — 1949

no significant TSH has been detected in any of the Cohn fractions Fraction II has been tested repeatedly at protein concentrations of from *1 3 to 8 per cent (2 7 to 16 mg per injection) Occasionally* borderline responses have been obtained with lower concentrations of Fraction II but higher concentrations have not shown any increase *in response Fraction III has proved highly toxic to animals on re* peated occasions and therefore has not been adequately tested Again surviving animals may show borderline responses but a two-dose response curve has been impossible to obtain because of toxicity of the fractions For this reason the variable responses observed at lower concentrations have been considered nonspecific Attempts to

separate the toxic factor from other substances of Fraction III have been unsuccessful

On the basis of these studies the cold ethanol method of fractionation seems unsuited for isolation of TSH from normal human sera

Returning to data reported in Table III Postel (1956) and Greenspan and Lew (1959) found no TSH activity in electrophoretically fractionated serum It is obvious however that TSH concentrations would have to be very great to be detected under the conditions used (minimum detectable TSH between 1 4 and 5 0 I mu per milliliter of serum) On the other hand D Angelo and Sunderman (1959) found TSH activity in alpha beta and albumin fractions but not in gamma globulin in pooled sera from normal individuals fractionated by flow electrophoresis The activity in the pooled fractions was approximately ten times that found in the Cohn fractions (Red Cross) In this respect it is interesting that in individuals with myxedema (McKenzie 1957b) as well as Graves disease (Adams and Purves 1957a) endogenous TSH was found to migrate with the γ globulins when the sera were fractionated electrophoretically Others (Lameyer *et al* 1955 Querido and Lameyer 1956) have reported endogenous serum TSH from patients with postoperative myxedema to be associated with Cohn fractions which migrate electrophoretically as β globulins To date the usefulness of flow electrophoresis in separating TSH activity from other serum proteins has been limited Further studies are required

Purves *et al* (1959) using ion exchange chromatography with carboxymethylcellulose (Condliffe and Bates 1956) followed by acetone fractionation a method shown to concentrate TSH in serum from myxedematous patients as well as to concentrated beef TSH added to sera were unable to detect TSH activity They conclude from this study that normal serum TSH content is less than 0 001 I mu per milliliter an extremely low value

Bates and Condliffe (1959) have obtained the most encouraging results to date in the isolation of TSH from normal human plasma Lyophilized plasma with Hyflo Super cel (Johns Manville) as a filter aid is suspended in 95 per cent ethanol and a cake formed in a sintered glass Buchner funnel This cake is successively extracted by a percolation procedure with 76 per cent ethanol/2 per cent NaCl 67 per cent ethanol/5 per cent NaCl 57 per cent ethanol/7 per cent NaCl and 38 per cent ethanol/8 per cent NaCl Maximum TSH activity is found in the 57 per cent ethanol/7 per cent NaCl percolate

After concentrating dialyzing and lyophilizing the combined 67 and 57 per cent ethanol NaCl fractions material was obtained which when assayed at three dose levels using a chick I^{131} depletion method (*Table I No 12*) *had a content of 0 5 I mu per milliliter plasma* equivalent and gave the same slope as the beef TSH standard D Angelo and Sunderman (1959) assaying the same material in stasis tadpole at two dose levels confirmed these findings Since approximately 50 per cent of TSH activity is lost in applying this fractionation procedure to pituitary powder Bates and Condliffe (1959) estimate a TSH content of 0 5 to 1 0 I mu per milliliter in *normal human plasma While this most exciting work will need* confirmation by others it appears to offer a very promising approach to the isolation of TSH from human sera

If these interesting findings are accepted a contradiction must be explained—i e the inability to measure TSH regularly in unfractionated sera when sera fractionated by the method of Bates and Condliffe (1959) contain 0 5 to 1 0 I mu per milliliter Certainly several assays are available with sufficient sensitivity to detect easily this concentration of TSH activity in unfractionated serum Postel (1956) has presented evidence suggesting that human sera contains substances which interfere with the bioassay of TSH and Greenspan *et al* (1957) and Starr *et al* (1958) have reported similar interfering *substances in urine Greenspan and Lew (1959) have recently reviewed* this problem and reported additional information on the interfering substance in urine While they have been unsuccessful in attempts to separate the interfering substance from TSH in urine (the TSH being either endogenously present or exogenously added) they have shown it to be nonhypophyseal in origin Whether or not such a factor exists in human sera is still debatable but should it be present it might explain the differences in TSH observed with and without serum fractionation

In an effort to confirm the studies reported by Postel (1956) as well as to determine if binding of TSH to serum proteins could be demonstrated a crude saline extract of post mortem human pituitary glands prepared by homogenizing frozen glands in 0 15 M NaCl buffered at pH 7 4 and removing the residue by centrifugation was subjected to electrophoresis on paper at pH 7 4 ionic strength 0 1 at 30 milliamperes for 18 hours both before and after mixing with 1 ml of normal human serum The results are shown in Figure 1 (Antoniades and Crigler 1959) *Unlike the findings reported by*

Postel (1956) who obtained very little migration of the human pituitary TSH (peak activity at the origin) TSH activity of the crude extract was found to migrate with the β 1 globulins its migration not being significantly altered by addition to this extract of a small

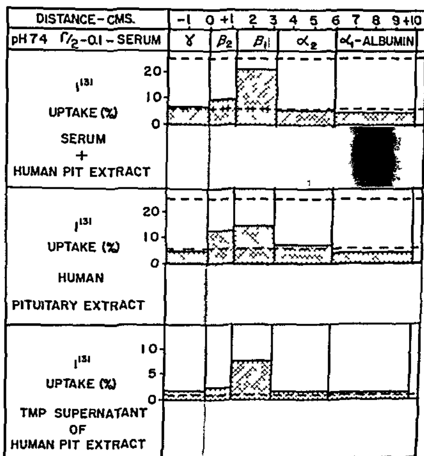


FIGURE 1 Paper zone electrophoresis of crude and purified human pituitary extracts with and without the addition of human plasma (From Antoniades and Crigler 1959)

amount of human serum In these studies none of the electrophoretic fractions interfered significantly with the bioassay Purification of the human pituitary extract by precipitation of inactive protein with tetrametaphosphate (Antoniades and Crigler 1959) did not alter its electrophoretic mobility in this system (Figure 1)

Postel s (1956) studies would suggest that there is both a pituitary and serum substance migrating electrophoretically on starch in a similar manner which can interfere with I^{131} uptake in chicks No

such pituitary or serum substance has been demonstrated by these studies although the quantities of serum may have been too small to detect the effect This is suggested by the previously mentioned work indicating that Cohn Fractions I and II + III in sufficient concentrations will suppress I^{131} uptake in chicks On the other hand D Angelo and Sunderman (1959) using the stasis tadpole assay have *found no inhibition of USP Thyrotropin Standard when administered* conjointly with fractions of normal serum obtained by flow electrophoresis For the present therefore a non iodinated substance capable of interfering with TSH assay has not been invariably found in spite of the fact that such a hypothesis is attractive and would explain the discrepancies between TSH content of unfractionated and fractionated sera

THYROTROPIC HORMONE(S) IN SERA FROM PATIENTS WITH ABNORMAL THYROID STATES

The role of the pituitary in regulating thyroid function has recently been reviewed by D Angelo (1954) For the past 30 years attempts have been made to determine TSH content of sera from patients with thyroid disorders in an effort to establish the primary or secondary role of this hormone in these diseases In the last few years with more sensitive and accurate biological assay methods findings from different laboratories have become increasingly consistent These *findings are summarized in the* following paragraphs

Thyrotropic Hormone(s) in Hypothyroidism (Congenital Spontaneous or Induced)

Table V lists the results obtained by various investigators who have assayed sera obtained from hypothyroid patients Hypothyroidism in these patients has been present congenitally occurred spontaneously or been induced by surgical excision or by medical treatment with radioactive iodine or antithyroid compounds While most observers have detected TSH activity in the sera of some hypothyroid individuals TSH has not invariably been found by any investigator This inability to measure TSH in the sera of certain patients with hypothyroidism has been tentatively explained on the basis of pituitary failure *secondary to long standing severe myxedema (Starr et al* 1939 D Angelo *et al* 1951 Gilliland and Strudwick 1956 DiGeorge *et al* 1957) *These workers support this hypothesis* by reporting initial

increases in serum TSH activity after treatment of some hypothyroid patients with thyroid hormone Since only a small number of such observations have been published further quantitative studies are

TABLE V TSH in Sera of Hypothyroid Patients

A tho s	Type f dis as	Ag y	N f p ti ts t sted	B y: Animal	M as ed espo	V l p r animal (ml)	Min TSH detectabl (I.m /ml)	E t TSH m an/ ang I.m /ml
Aron et al (1931 1932 1933)	—	—	—	guinea pig	h sto	15	0 6 1 0	detected
Hertz and Oastler (1936)	spont.	adults	9	hypox at	histo	10 20	0 75	detected
DeRob tis (1948)	spont induced	27 28	2	guinea pig	colloid droplet	2	0 01	1 25
P r s a d G ie bach (1949)	pont.	—	5	guinea pig	histo	10 0	1 0	d tected
D Angel et l (1951)	cong spont.	11 29 68	1 9	sta l tadpole	histo	0 35	0 5	0 9 0 3 8
Simki et al. *(1952)*	spont	—	8	sta i *tadpole*	hi to	—	—	detected
Asboe Ha on et al (1952)	—	—	7	stasis tadpole	histo	0 18	1 7	ot detected
G lili d a d Str dw k (1956)	spo t.	48 63	10	h ck	I^{131} depletio	2 4	0 25	0 9 0 1 9
	ong	12 17	2	chick	I^{131} depleti n	2 4	0 25	1 7 2 6
	nd ced	38 42	2	chick	I^{131} depleti	2 4	0 25	1 6 2 8
D Ge ge et al. (1957)	c g	5/12 11	5	ta l tadpole	h sto	0 35	0 5	0 9 0 3 6
Ad ms (1958)	co g	1 11	4	gui ea pig	I^{131} d pl t o (bl)	1	0 1	2 0 1 2 5
McKenzie (1958b)	—	—	6	m	I^{131} depl t o (bl)	0 5	0 1	0 31 0 12 0 64
G nspa a d Lew (1959)	po t.	50 55	4	chi k	I^{131} d pl t o	2	1 0	d tect d
C gl t al (1959)	co g	3/12 5	3	m	I^{131} depl t on (bl)	0 5	0 3	1 0 3 7
	p t	11	1	m e				

E tim t d ing al ti f as y as publi h d l t (D Geo g t l 1957)

Abb tions po t. po taneo ng ng nital bl blood

necessary to exclude other possible explanations for the variable TSH activity in the sera of hypothyroid patients

With the exception of the single very high value reported by DeRobertis (1948) all other investigators obtained values of TSH activity between 0 and 3 8 I mu per milliliter in sera from hypothy

roid individuals While the high level reported by DeRobertis (1948) could have been a nonspecific bioassay response (Dvoskin 1947 1948) or the result of fractionation of the serum by acetone precipitation no interpretation seems justified since it represents an isolated observation The inability of Asboe Hansen *et al* (1952) to detect TSH activity in hypothyroid sera using the stasis tadpole assay is best explained by the concentration of TSH required for detection when such small quantities of sera are injected

Since TSH has not been unequivocally measured in normal sera or in sera from all individuals with primary hypothyroidism the secondary origin of hypothyroidism (hypopituitarism) can hardly be established at this time by the finding of low or absent serum TSH concentrations Nevertheless it should be mentioned that two groups of workers (D Angelo *et al* 1951 Gilliland and Strudwick 1956) who report detectable TSH in some normal sera have failed to measure TSH in the sera of individuals with known hypopituitarism

Thyrotropic Hormone(s) in Hyperthyroidism (Graves Disease)

Soon after it had been clearly established that the anterior pituitary gland secretes a hormone which stimulates the thyroid gland (Smith and Smith 1922 Smith 1927 Uhlenhuth and Schwartzbach 1928 Loeb and Bassett 1929 1930 Aron 1929 1930 Schockaert and Foster 1932 Closs *et al* 1932) speculation concerning a possible role of TSH in the etiology of Graves disease was raised Harrington (1933) considered that an increased secretion of TSH by the anterior pituitary gland might be the cause of hyperthyroidism but was careful to emphasize that there was no evidence at that time to support this suggestion

Table VI summarizes the results obtained by different investigators who have studied the TSH content of sera from individuals with Graves disease in various states of thyroid activity and with and without significant eye signs DeRobertis (1948) first detected TSH in sera from hyperthyroid individuals but only in those individuals with exophthalmos The sera assayed were fractionated by acetone precipitation according to the method described by Fellinger (1936) Subsequently a number of investigators (see Table VI) assaying unfractionated serum have confirmed DeRobertis findings In general these limited observations suggest that TSH activity is not significantly elevated in untreated hyperthyroid individuals On the other hand untreated thyrotoxic patients with exophthalmos more consistently

have detectable serum TSH activity at times TSH concentration seems to be significantly increased although usually less than that observed in hypothyroid subjects Patients with Graves disease with

TABLE VI TSH in Sera of Patients with Graves Disease

Author	Clinical status			No. of samples tested	Bioassay		Vol. per animal (ml.)	Min. TSH detectable (I.mu./ml.)	Est.* TSH mean/range (I.mu./ml.)
	Thyroid function	Eye signs	Treatment		Animal	Measured response			
Aron (1931, 1932, 1933)	hyper	min.	no	—	guinea pig	histo	15	0.6–1.0	not detected
Aron et al. (1931, 1932)									
Hertz and Oastler (1936)	hyper	—	no	7	hypox. rat	histo	10–20	0.75	not detected
Fellinger (1936)	hyper	—	no	48	guinea pig	histo	15–30	1.0	not detected
Cope (1938)	hyper	—	no	2	guinea pig	histo	10	1.0	not detected
DeRobertis (1948)	hyper	min.	no	4	guinea pig	colloid droplet	2	0.01	not detected
	hyper	exoph.	no	4	guinea pig	colloid droplet	2	0.01	detected
Purves and Griesbach (1949)	hyper	min.	no	22	guinea pig	histo	10.0	1.0	not detected
	eu	min.	yes	19	guinea pig	histo	10.0	1.0	detected
	hyper	exoph.	no	37	guinea pig	histo	10.0	1.0	detected
D'Angelo et al. (1951)	hyper	—	no	8	stasis tadpole	histo	0.35	0.5	0.6 0–1.5
	eu to hypo	exoph.	no	6	stasis tadpole	histo	0.35	0.5	1.1 0–3.9
Simkin et al. (1952)	hyper	min.	no	—	stasis tadpole	histo	—	—	not detected
	hyper	exoph.	—	—	stasis tadpole	histo	—	—	? detected
Asboe-Hansen et al. (1952)	hyper eu	exoph. exoph.	yes yes	5 5	stasis tadpole	histo	0.18	1.7	4.3 0–10.4
Gilliland and Strudwick (1956)	hyper	min. to mod.	no	5	chick	I^{131} depletion	—	0.2	0.17 0–.47
	hyper	severe	no	8	chick	I^{131} depletion	—	0.2	0.5 0–1.0
		severe	no	3	chick	I^{131} depletion	—	0.2	not detected
Adams (1958)	hyp	exoph.	no	5	guinea pig	I^{131} depletion (BI)	1.0	0.1	detected in 3†
McKenzie (1958a)	hyp	exoph.		11	mouse	I^{131} depletion	0.5	0.1	detected in 9†

* Estimated using all units of assay as published later (DiGeorge et al., 1957)
† Showing prolonged effect with delayed maximum response

or without exophthalmos made hypothyroid by surgical or medical treatment usually have increased concentrations of TSH activity as mentioned in the previous section

Evidence for an Abnormal Thyrotropic Hormone in Patients with Graves Disease

Adams and Purves (1957a) after summarizing the available information on TSH content of biological fluids in different thyroid states discussed the role of TSH in hyperthyroidism and exophthalmos They suggested that sera from patients with Graves disease may contain an abnormal form of TSH which is responsible both for exophthalmos and hyperthyroidism Using guinea pigs pretreated with I^{131} and l thyroxine in which TSH response is measured by determining the peak increase in blood I^{131} occurring 2 to 3 hours after injection of TSH (Table I No 10) Adams and Purves (1956) and Adams (1956) found that serum from some patients with Graves disease gave a peak response 14 to 22 hours after that obtained by the TSH standard Subsequent studies by Adams (1958) showed that this abnormal response was not produced by extracts of rat mouse or human pituitary glands or by sera from thyroidectomized rats or myxedematous human beings all of which gave the normal peak response 2 to 3 hours after injection He also found that the abnormal response was still given by the sera of some hyperthyroid individuals after they had been rendered euthyroid by I^{131} or surgery and was not suppressed in individuals with untreated Graves disease when thyroxine was administered In addition when the beef pituitary TSH standard was added to a serum giving the abnormal response and the mixture assayed the magnitude and time course of the response given demonstrated that the two components acted independently and simultaneously This indicated that the abnormal response was not apparently due to the presence of some substance in the serum which modifies the time course of action of normal TSH When serum containing the abnormal thyroid stimulator was subjected to electrophoresis the abnormal response was given by the γ globulin fraction (Adams and Purves 1957b) Recently Purves *et al* (1959) have described a protein component of sera from 5 patients with untreated Graves disease which gives a significant abnormal response This protein component which migrates electrophoretically like the serum γ globulins was obtained using a carboxymethylcellulose column in a manner described for pituitary TSH purification by Condliffe and Bates (1956) A similar concentrate from a patient with toxic nodular goiter and no exophthalmos gave no response

McKenzie (1958a) using a similar assay which employed mice instead of guinea pigs confirmed the presence of the long acting thyroid stimulator in thyrotoxic individuals with and without exophthalmos

Further study of these interesting observations is indicated

SUMMARY

Available information concerning the content and nature of TSH in human sera has been reviewed Although the sensitivity accuracy and convenience of TSH bioassays have been improved by the application of appropriate statistical design and by the introduction of radioactive isotopes biological assay still represents a significant limiting factor in the study of TSH in serum and urine That this is not the only difficulty however is evident from the study of unfractionated and fractionated normal human sera TSH has not been *unequivocally found in unfractionated normal sera although bioassays* exist capable of detecting as little as 0 1 I mu per milliliter with great significance While results obtained with fractionation by acetone precipitation the methods of Cohn and co workers flow electrophoresis and column chromatography with various materials have proved unsatisfactory for concentrating TSH activity from serum a new method of fractionation involving serial extraction of lyophilized serum with different ethanol NaCl solutions (decreasing in ethanol concentration and increasing in sodium chloride concentration) (Bates and Condliffe 1959) seems most promising Using this method of isolation TSH has been found in normal serum in con*centrations of 0 5 to 1 0 I mu per milliliter This amount* of TSH should be easily detected in unfractionated sera by a number of bioassay methods although this has not been the case The apparent contradiction in TSH content of unfractionated and fractionated serum may have important implications with respect to the chemical state of TSH in human serum and therefore with respect to its physiological effects

Limited studies of TSH activity of sera from individuals with abnormal thyroid function indicate that TSH may be increased in primary hypothyroidism whether of unknown etiology or surgically or medically induced In untreated hyperthyroidism of Graves disease serum TSH content would seem to be low normal or high making it impossible by this means to establish firmly an etiological role for

TSH in this disease The newer observation of an abnormal TSH in sera of individuals with Graves disease (Adams 1958 McKenzie 1958a) perhaps offers an explanation for the discrepancies observed in the previous studies of thyrotoxic serum

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CHAPTER IX

*Vasopressin and Oxytocin in the Plasma of Man and Other Mammals**†

Henry D Lauson

CHEMISTRY OF THE NEUROHYPOPHYSIAL HORMONES
- The octapeptides
- The van Dyke protein

PHYSIOLOGICAL AND PHARMACOLOGICAL ACTIONS
- Antidiuretic action
  - Renal concentrating and diluting processes
  - Limits of the concentrating and diluting processes
  - Factors which modify the normal limits
    - Change in GFR
    - Influence of previous state of bodily hydration on the concentrating process
    - Influence of level of protein in the previous diet on the concentrating process
    - Adrenal cortical hormones
    - Potassium depletion
    - Age
    - Renal diseases
- Vasopressor action
- Oxytocic action
- Milk ejection
- Avian depressor action

NATURE OF THE CIRCULATING HORMONES

ENTRY INTO THE BLOOD
- Formation
- Liberation
  - The simultaneous release concept
- Natural stimuli for release of antidiuretic hormone

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It is the purpose of this chapter to summarize current knowledge pertaining to the neurohypophysial hormones in human plasma Because very little is known concerning this immediate topic it will be necessary to draw heavily on information obtained from experiments on other mammals particularly the rat and the dog A basis will be carefully developed for predicting with reasonable approximation the physiological range of concentration of these hormones in man This indirect approach is necessary because the concentrations are so exceedingly small that no existing bioassay or chemical method is sufficiently sensitive and specific to permit direct measurement

CHEMISTRY OF THE NEUROHYPOPHYSIAL HORMONES

The unitary hypothesis concerning the chemical nature of these hormones proposed and persistently defended by Abel (1930) attributed all activities of extracts of posterior pituitary glands to a single substance The dual hypothesis was proposed by Dudley (1919) and convincingly supported by Kamm and associates (1928) who effected a considerable separation of two fractions pressor antidiuretic and oxytocic from acidified and boiled posterior pituitary tissue At the present time interestingly enough the essential features of both hypotheses still present valid possibilities despite the brilliant achievements of du Vigneaud and associates (1953a 1953b 1953c 1954a 1954b 1954–55) in isolating purifying determining the structure and finally synthesizing the octapeptides oxytocin and vasopressin

The Octapeptides

These are the minimum molecules responsible for the vasopressor antidiuretic oxytocic avian depressor and milk ejection activities of mammalian neurohypophysial extracts Oxytocin and the vasopressins consist of a pentapeptide disulfide ring with a tripeptide side chain (Fig 1) Six amino acids are common to both three being amides Cystine tyrosine glutamine and asparagine are in the same position in both rings and proline and glycine amide are common to both side chains Oxytocin has isoleucine in the ring and vasopressin contains phenylalanine in this position Leucine in the side chain of

NORMAL RATES OF LIBERATION OF VASOPRESSIN AND OXYTOCIN
- Constant infusion of antidiuretic hormone
  - Data on human beings
  - Data on dogs
  - Data on rats
- Constant infusion of oxytocic hormone
- *Single* intravenous injections of antidiuretic hormone
  - Rapidity of ADH release and onset and persistence of its renal effect
  - Gradation of response to single injections
  - Estimation of normal concentrations of ADH in plasma
  - Estimation of the rate of removal of ADH from the plasma
- Single intravenous injections of oxytocin

SITES OF REMOVAL OF THE HORMONES FROM THE PLASMA
- Inactivation of ADH by the blood
- *Removal of ADH by liver and kidneys in rats*
- Removal of ADH by liver in dogs
- Removal of ADH by kidneys in dogs
- Removal of vasopressin by muscle
- *In vitro* studies
- Removal of oxytocin from the circulation

ESTIMATION OF NORMAL PLASMA CONCENTRATIONS
- Theoretical considerations
  - During a constant rate of secretion (or infusion)
  - After abrupt cessation of secretion (or infusion)
  - Validity of the assumptions and the resulting equations
  - Experimental application of the equations
- Estimation of the removal rate coefficient C/V for antidiuretic hormone
  - *Estimation of C/V for ADH in man*
    - Data of Hollander *et al* (1957a) on normal subjects
    - Data of Lauson (1951) on one normal human subject
  - Estimation of C/V for ADH in dogs
  - Estimation of C/V for ADH in rats
- Prediction of the physiological ranges of antidiuretic hormone concentration in plasma
- Estimation of plasma concentrations and C/V for oxytocin in women during parturition

BIOASSAY OF ENDOGENOUS VASOPRESSIN AND OXYTOCIN IN BLOOD PLASMA OR SERUM
- Bioassay methods
- Results of bioassays of blood plasma or serum for antidiuretic activity
  - Physiological dehydration
  - Other conditions
- Results of bioassay of blood for oxytocic and milk ejecting activities

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as an antidiuretic agent in hydrated young pigs than is the arginine form in the hydrated dog or rat arginine vasopressin is the more effective Sawyer (1958) showed *qualitative* differences in the anti diuretic effects of these two vasopressins in rats

Table I taken from the data of van Dyke (1959) compares oxy tocin and the two vasopressins with respect to their oxytocic avian depressor milk-ejection vasopressor and antidiuretic activities

TABLE I Relative Potencies of Highly Purified Oxytocin and the Vasopressins†

Substance	In vitro		Intravenous			
	Oxytocic (isolated rat uterus)	Oxytocic (isolated hen uterus)	Vasodepressor (fowl)	Milk ejection (rabbit)	Vasopressor (rat)	Antidiuretic (dog)
Oxytocin‡	100	8	100	100	2	1
Arginine vasopressin§	5	80	14	17	100	100
Lysine vasopressin‖	5?	5	14	17	100	17

For each substance the assay which resulted in the greatest potency was assigned the value of 100

†Adapted from van Dyke (1959)

‡The value of 100 corresponds to maximum absolute potency of the order of 450 to 500 units (based on the new U S P Standard Posterior Pituitary Powder) per milligram of highly purified oxytocin.

§The value of 100 corresponds to an absolute potency of about 400 to 450 U S P units per milligram of highly purified arginine vasopressin.

‖The value of 100 corresponds to an absolute potency of about 275 to 325 U S P units per milligram of highly purified lysine vasopressin

The van Dyke Protein

Abel s unitary hypothesis received support from Rosenfeld (1940) who ultracentrifuged chilled fresh press juice from bovine posterior lobes and found that oxytocic and pressor activity sedimented as if both were part of one or more protein molecules Heating of the juice in acid solution resulted in a decreased sedimentation as if smaller active fragments had been cleaved from a parent molecule Van Dyke *et al* (1942) isolated a protein (molecular weight 30 000) from freshly frozen beef posterior lobes with apparently constant physicochemical properties and containing all the biological activities of posterior pituitary extracts in about the same proportions as they exist in the U S P reference standard The yield of their protein was small and much of the initial activity was dialyzable Post mortem autolysis of the native protein could possibly account for the latter phenomenon

Recently Acher and Fromageot (1957) have summarized their extensive studies of the bovine van Dyke protein They observed that the octapeptides dissociated from the parent protein during

oxytocin is replaced by arginine in beef vasopressin and by lysine in hog vasopressin One amino group of cystine is free in both hormones The molecular weights of oxytocin arginine and lysine vasopressins are 1007 1084 and 1056 respectively

Oxytocin in beef and hog posterior pituitary lobes has the same structure Light and du Vigneaud (1958) recently showed that human oxytocin also has this structure Proof of structure is not available for oxytocin of other mammalian species According to van Dyke *et al*

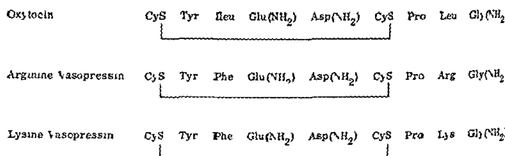


FIGURE 1 Structure of the natural mammalian neurohypophysial hormones (From du Vigneaud *et al* 1953a c)

(1955) the pure octapeptide inherently possesses vasopressor and antidiuretic properties but only to a very small extent

Mammalian vasopressin exists in two known forms as previously mentioned The more ubiquitous *arginine vasopressin* has been established chemically as the natural hormone in the bovine (du Vigneaud *et al* 1953a) and human species (Light and du Vigneaud 1958) and is believed on the basis of pharmacological criteria to be the natural hormone also in the macaque monkey dog rat rabbit sheep and camel (van Dyke and associates 1956 1957)

Lysine vasopressin (du Vigneaud *et al* 1953a) differs from the arginine form only in that lysine replaces arginine in the penultimate position It is the natural hormone in swine and so far has not been found in any other species

Both vasopressins in pure form either natural or synthetic exhibit some degree of oxytocic avian depressor and milk ejection properties the ratios of these potencies to that of their vasopressor activity are the same in the two vasopressins according to van Dyke *et al* (1956) There seems to be no valid evidence to indicate that antidiuretic and vasopressor effects are caused by separate compounds Munsick *et al* (1958) found that lysine vasopressin is as potent or even more potent

as an antidiuretic agent in hydrated young pigs than is the arginine form in the hydrated dog or rat arginine vasopressin is the more effective Sawyer (1958) showed *qualitative* differences in the anti diuretic effects of these two vasopressins in rats

Table I taken from the data of van Dyke (1959) compares oxy tocin and the two vasopressins with respect to their oxytocic avian depressor milk-ejection vasopressor and antidiuretic activities

TABLE I Relative Potencies of Highly Purified Oxytocin and the Vasopressins†

Sub tanc	In itro		Intra eno			
	Oxyto i (l ted t t ru)	Oxytocic (isol ed h terus)	Vaso- dep s (f l)	Milk jec on (rabb t)	Vas p es (t)	Ar ti diur ti (dog)
Oxytoci ‡	100	8	100	100	2	1
Arg in a p ss n§	5	80	14	17	100	100
Ly sopr in‖	5?	5	14	17	100	17

F h ubstanc th as ay wh h ul ed i th gr tes pote y as gned h l f 100

†Adapted from an Dyk 19 9

‡Th al f 100 corr ponds to maxim m absol pote cy f th d f 450 to 500 units (b ed on th new U S P Standard P t io Pituitary Powd) p millig am f highly pu ified ytocin.

§Th al f 100 po ds to an absol te potency f abo t 400 to 450 U S P units p millig am of h ghly purified rgir as p in

‖The al f 100 ponds to ab l t po ency f abou 275 to 325 U S P units p millig am of highly purified lysin asopres in

The van Dyke Protein

Abel s unitary hypothesis received support from Rosenfeld (1940) who ultracentrifuged chilled fresh press juice from bovine posterior lobes and found that oxytocic and pressor activity sedimented as if both were part of one or more protein molecules Heating of the juice in acid solution resulted in a decreased sedimentation as if smaller active fragments had been cleaved from a parent molecule Van Dyke *et al* (1942) isolated a protein (molecular weight 30 000) from freshly frozen beef posterior lobes with apparently constant physicochemical properties and containing all the biological activities of posterior pituitary extracts in about the same proportions as they exist in the U S P reference standard The yield of their protein was small and much of the initial activity was dialyzable Post mortem autolysis of the native protein could possibly account for the latter phenomenon

Recently Acher and Fromageot (1957) have summarized their extensive studies of the bovine van Dyke protein They observed that the octapeptides dissociated from the parent protein during

dialysis against dilute acetic acid during Tiselius electrophoresis at pH 4 4 during electrodialysis and during precipitation with 5 per cent trichloracetic acid Cleavage did not occur during dialysis against water Cysteine failed to destroy either oxytocic or vasopressor activity of the protein the same is true of Pitressin and Pitocin (*Sealock and du Vigneaud 1935*) *Trypsin destroyed the vasopressor* activity of the protein but left oxytocic potency unchanged Trypsin is known to inactivate pure vasopressin but not oxytocin (Lawler and du Vigneaud 1953)

Each molecule of the van Dyke protein apparently consists of one oxytocin and one vasopressin molecule attached to an inert protein Haselbach and Piquet (1952) suggested that the active polypeptides are held to the protein by electrostatic forces Acher and Fromageot (1957) consider adsorption as another possibility and cannot at present decide if the van Dyke protein is an artifact of the extraction process or a substance of biological importance

It is a matter of considerable importance that Acher (1958) has reported preparation of a protein from posterior lobes of hogs which is similar to the bovine protein of van Dyke *et al* (1942) with respect to sedimentation and diffusion constants nitrogen content and oxytocic and vasopressor activity per milligram Furthermore the active fragments can be dissociated by the same methods found by Acher and Fromageot (1957) to be effective in the bovine protein However Kiessling and Elmqvist (1958) have subjected freshly frozen hog posterior pituitary glands to mild fractionation procedures and were able to account for little if any of the activity in the form of a van Dyke protein They believe the activities are associated with a variety of labile molecules

No definite conclusions seem possible at present regarding the chemical form of the octapeptides as they exist in the neurohypophysis Furthermore there are no certain answers as yet to the important physiological questions as to whether release of the hormones into the blood involves transport of a molecule like the van Dyke protein whether the octapeptides are released as such into the blood or indeed whether some other carrier becomes involved

PHYSIOLOGICAL AND PHARMACOLOGICAL ACTIONS

Antidiuretic Action

Renal Concentrating and Diluting Processes The essential action of ADH on the kidney is believed to be that of increasing the

permeability to water of the distal convoluted tubule Water is thereby enabled to move passively from lumen to interstitial fluid along an osmotic gradient This kind of permeability increasing action of ADH was first shown by Koefoed Johnsen and Ussing (1953) on the skin of frogs and toads Were no other mechanisms available the most that could be achieved by ADH would be the production of urine isosmotic with plasma This indeed is the effect of ADH in water loaded frogs and toads previously hypo-osmotic urine becomes isosmotic and re duced in volume (Sawyer 1957a b) Moreover urine somewhat hyper osmotic to plasma can be excreted by dogs (Shannon 1942a Berliner and Davidson 1957) and man (Kleeman *et al* 1957) under certain conditions in the *absence* of ADH Therefore elaboration of con centrated urine seems to require first the anatomical arrangement provided in the medulla of birds and mammals by the loops of Henle the capillary loops (vasa recta) and the unidirectional collecting ducts which emerge at the apex of the papilla and second the cellular mechanisms of active sodium (chloride) transport and variable per meability of the tubular epithelium to water and sodium (chloride)

Through micropuncture techniques the following facts have been established concerning the osmolar concentration of fluid in the lumen of the mammalian tubule (1) In the proximal convoluted tubules the fluid is isosmotic with the plasma (Walker *et al* 1941 Wirz 1956) (2) At the bend of the loop of Henle it is hyperosmotic to plasma (Gottschalk and Mylle 1959) (3) Early in the distal convoluted tubule on the other hand the fluid is hypo-osmotic whether the final urine is hypo-osmotic (Wirz 1957) or hyperosmotic (Wirz 1957 Gottschalk and Mylle 1959) (4) Along the distal convoluted tubule in the pres ence of sufficient ADH osmolar concentration increases to but not beyond isotonicity with plasma (Wirz 1957 Gottschalk and Mylle 1959) in the absence of ADH the fluid in the distal tubule remains hypo-osmotic (Wirz 1957) (5) One additional fact is of major im portance there is a gradient of osmolar (or sodium chloride or urea) concentration in the medullary interstitial fluid from the cortico medullary border where it is isosmotic with plasma to the apex of the papilla where it is strongly hyperosmotic to plasma (for references see Gottschalk and Mylle 1959)

As these important data have accumulated in recent years hypotheses concerning the concentrating and diluting mechanisms have been elaborated and several times modified The following summarizes the current views set forth in the outstanding paper of Gottschalk and Mylle (1959 see also Wirz 1957 and Berliner *et al* 1958) (1) A major

portion of the filtered sodium and associated anions is reabsorbed isosmotically in the proximal convoluted tubules by means of active transport of sodium and passive movement of water (Walker *et al*, 1941) (2) Fluid descending within the thin segment of the loop of Henle becomes increasingly hyperosmotic (compared to systemic plasma) as it tends to equilibrate with surrounding interstitial fluid possibly by passive inward diffusion of sodium (chloride) and urea and by passive outward diffusion of water (3) Conversely the cells of the ascending limb of the loop of Henle are assumed to transport sodium actively from lumen to interstitial fluid while water tends to be retained within the lumen because of a postulated relative water impermeability *in this segment Such an arrangement would provide* both for the graded elevation of sodium concentration in the medullary interstitial fluid (countercurrent multiplier mechanism of Hargitay and Kuhn 1951 as modified by Wirz 1957 and Gottschalk and Mylle 1959) and for the hypo osmolar concentration of the fluid entering the distal convoluted tubule (4) In the absence of ADH the distal convoluted tubule apparently is relatively impermeable to water *so that the hypo osmolarity of the fluid is maintained and per* haps augmented by the continuing active reabsorption of sodium (chloride) in this segment In this circumstance a large flow of dilute fluid is delivered to the collecting ducts When these cortically located distal convoluted tubules are under the influence of sufficient ADH however water diffuses outward from the lumen Diffusion continues until the osmotic gradient previously established in the fluid by the *activity of the cells of the ascending limb and continued* by the further active outward transport of sodium (chloride) in the distal tubule is dissipated By these means the fluid in the tubule becomes isosmotic and the rate of flow into the collecting ducts is greatly reduced The major portion of water conservation in antidiuresis takes place in the distal convoluted tubules as emphasized by Berliner *et al* (1958) (5) In the presence of sufficient ADH as the reduced volume of fluid courses along the water permeable collecting duct hyper osmolarity develops due to passive loss of a small volume of water and passive transfer of urea the final urine is as concentrated as the apical interstitial fluid Permeability to water in these ducts is believed to be enhanced by ADH (Berliner *et al* 1958) It is interesting to note that Smith (1951) and Wesson and Anslow (1952) prior to the micro puncture studies of Wirz (1956 1957) and of Gottschalk and Mylle (1959) deduced from their own observations and those of Rapoport

et al (1949) that the concentrating process was located far distally in the nephron perhaps even in the collecting ducts which up to that time had been generally regarded as mere conduits (See Berliner *et al* 1958 and Levinsky and Berliner 1959 for their interesting discussion of the special role of urea in the concentrating process) In the absence of ADH the fluid entering the collecting ducts is much larger in volume per minute and is still hypo osmotic Loss of a relatively small volume of free water in the collecting ducts would have little effect on the final urinary osmolarity and flow rate

Of major importance in the maintenance of the gradient of hyper osmolarity in the medullary interstitial fluid is the anatomical arrange ment of medullary capillaries (vasa recta) These vascular loops are intermingled with the loops of Henle and like other capillaries tend to equilibrate rapidly with surrounding interstitial water and solutes The blood during descent into the medulla therefore gains in osmo larity and near the bend of the vascular loop the blood has the same osmolar concentration as the fluid in the bend of adjacent loops of Henle (Gottschalk and Mylle 1959) During ascent the blood osmolar ity returns toward normal by losing most of the sodium (chloride) and urea acquired during descent and by regaining water In the process the hyperosmolarity of the medullary interstitial fluid is largely pre served Even though a fairly large blood flow may pass through these capillaries the net removal of sodium (chloride) and urea from this interstitial is kept low because these vessels function as countercurrent diffusion exchangers (Wirz 1953 Berliner *et al* 1958 Gottschalk and Mylle 1959)

In brief ADH is believed to increase the permeability to water of the epithelium of the distal convoluted tubules (and perhaps also that of the collecting ducts) The increase in permeability is graded when the concentration of ADH in plasma is graded When the permeability is increased maximally the osmolar concentration of the fluid leaving the distal convoluted tubules has increased from hypo to isotonic and the flow of this fluid has been greatly decreased Diffusion from this reduced volume of a small quantity of free water in the collecting ducts effects an increase from iso to hypertonicity of the final urine In terms of the quantity of water conserved the direct effect of ADH on the permeability of the distal tubules is quantitatively far more important than the final concentrating process

Limits of the Concentrating and Diluting Processes The conse quences relevant to the primary subject of this chapter of the

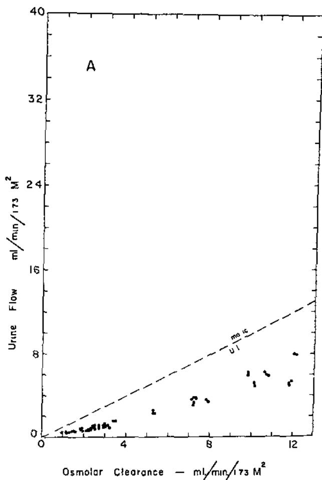
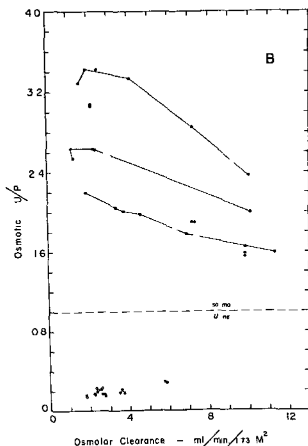


FIGURE 2 *A* Minimum (overnight dehydration plus Pitressin) and maximum (water diuresis) urine flows as a function of osmolar clearance in normal human subjects and patients with diabetes insipidus (X) The solid circles below the isosmotic urine line represent data from individual periods (unpublished) of Boyarsky and Smith (1957) Above the line the solid circles represent average values from the control periods of all subjects except W M of Hollander *et al* (1957a) open circles represent selected average data of Kleeman *et al* (1956) open triangles indicate average control values of Raisz *et al* (1957) X individual data from 4 patients with diabetes insipidus (excluding patient S) of Heineman and Becker (1958) On the ordinate the vertical distance from zero to the isosmotic urine line represents the osmolar clearance component of urine flow The vertical distance from the isosmotic line to the plotted points represents the free water clearance component of urine flow (positive above the isosmotic line and negative below this line)

processes summarized above are perhaps best shown in graphs based on data from normal human subjects (Fig 2) The volume of isotonic fluid leaving the proximal tubules per minute *glomerular filtration rate (GFR) being held constant* varies in proportion to the quantity



B Maximum and minimum osmotic *U/P* as a function of osmolar clearance Symbols are the same as in *A* but here the data of Bojarsky and Smith (1957) are above the isosmotic line Consecutive values from three of their subjects have been connected by lines to indicate the trend in individual subjects

of osmotically active substances left unabsorbed Thereafter whether sufficient ADH is present in the plasma to induce a maximum anti diuretic effect or whether no ADH is present the volume per minute of the final urine is determined in part by the extent to which solutes remain unabsorbed (as estimated from the final rate of excretion of total solutes) A convenient way of representing this was developed by Smith (1951 1955) and Wesson and Anslow (1952) The clearance of osmotically active solutes (C_m in milliliters per minute) is the rate of excretion of total solutes (U_mV in micro osmols per minute) divided by the plasma osmolar concentration (P_m in micro osmols per milliliter) C_m represents the volume which the total solutes

excreted in one minute would occupy if present in a solution iso osmotic with the plasma In the absence of ADH the equivalent of a large volume of free water (C_{H_2O} in milliliters per minute) remains unabsorbed and in effect is added to the C_{osm} Thus the total urine flow (V) can be thought of as made up of two components

$$V = C_{osm} + C_{H_2O}$$

When maximally concentrated urine is excreted C_{H_2O} is negative because free water has been conserved by the kidneys This moiety has been designated T_{H_2O} to denote the volume of free water trans ported per minute from tubule lumen to interstitial fluid in the con centrating portion of the nephron For purposes of the present discussion however the symbol C_{H_2O} is applied to the entire range of change in free water clearance

In the graphs shown in Figure 2A and B the abscissa represents *the osmolar clearance Experimentally changes in C_{osm} are usually* induced by intravenous administration of increasing amounts of a solute such as mannitol which has a high renal clearance Total urine flow partially dependent on C_{osm} is plotted on the ordinate in Figure 2A and osmotic U/P ratio (urine/plasma osmolar concentration ratio) is plotted on the ordinate in Figure 2B The full range of ADH action is represented by the vertical distance between the upper values of V generated during complete suppression of ADH release (achieved by adequate water loads) and the lower values of V resulting from supramaximal concentrations of ADH in the plasma Similarly the full range of ADH action in Figure 2B is represented by the vertical *distance between the upper and lower values of the osmotic U/P* ratio

Several points may be emphasized (1) In the absence of ADH as C_{osm} increases C_{H_2O} increases at first sharply and then more gradually The progressive increase in C_{H_2O} has been attributed by Orloff and associates (1956 1958) to a progressive decline in the rate of diffusion of freed water out of the lumen of the tubule as a consequence of the osmotic restraint of unabsorbed solute there is probably some degree of permeability to water in the distal convoluted tubules even in the absence of ADH (2) Under maximum ADH effect negative C_{H_2O} (i e T_{H_2O}) increases with increasing C_{osm} and at higher values of C_{osm} tends to become constant ($T^{c}m_{H_2O}$) (3) At the lowest values of C_{osm} the osmotic U/P ratios are at their lowest (in the absence of ADH) and at their highest (under maximum ADH) with increasing

C_{osm} both limiting values of U/P tend toward 1 0 (Rapoport *et al* 1949 Brodsky and Rapoport 1951) (4) From an examination of the unpublished data of Boyarsky and Smith* (1957) it is evident that in any one subject during maximum ADH action the values of U/P as a function of C_{osm} tend to trace a smooth curve parallel to the average trend most of the apparent variation seen in Figure 2B is among the subjects not within any one

Factors Which Modify the Normal Limits *Change in GFR* During an experiment or ADH bioassay change in GFR will operate mainly by changing C_{osm} If this were the only effect an adequate correction could be applied by dividing all values of V C_{H_2O} and C_{osm} by the concurrent values of GFR Unfortunately it is more complicated It was shown by Shannon (1942a) and more recently by Berliner and Davidson (1957) using superior techniques that if GFR is moderately reduced *in the absence* of ADH the urine can become slightly to moderately *hypertonic* (see also Lauson 1951 and Berliner *et al* 1958) Kleeman *et al* (1957) del Greco and de Wardener (1956) and Dicker (1957) demonstrated a similar phenomenon in man dogs and rats respectively Under these circumstances only a small addition of ADH would probably be required to produce maximal concentration of the urine Moreover Levinsky *et al* (1958) found that decrease of GFR by 10 to 25 per cent in *dehydrated* dogs further increased the already high osmotic U/P by as much as 40 per cent The factor of decreased GFR is undoubtedly of major importance in achieving true oliguria during dehydration (Shannon 1942a b) Dicker (1957) has come to similar conclusions regarding the rat It is probable that the more labile the GFR is in a species (rat > dog > man) the more important this factor becomes

It is less certain that an *increase* in GFR alters renal sensitivity to ADH other than through its effect on C_{osm}

Influence of previous state of bodily hydration on the concentrating process The most clear-cut and extensive data are those of Epstein *et al* (1957a) They measured V and maximum osmotic U/P during Pitressin infusion after 12 hours of dehydration in subjects on regimens of dehydration and forced hydration After previous dehydration the maximum osmotic U/P averaged 3 99 (3 37 to 4 48) for the range of C_{osm} of 1 05 to 3 55 ml per minute In striking contrast after previous forced hydration the maximum osmotic U/P averaged only 2 61 (2 21 to 3 02) for the range of C_{osm} of 1 37 to 4 10 ml per minute (compare

* These authors kindly made their unpublished data available to the reviewer

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GFR and C_m were unchanged from control values The well known impairment of water diuresis in adrenal insufficiency can be attributed mainly to decrease in GFR but the absence of glucocorticoids probably is responsible for a part of the effect Whether changes in the plasma concentration of adrenal steroids alter the renal response to ADH (i e change the kidney sensitivity to ADH) other than through an effect on GFR and C_m is not established at this time because of the many difficulties in conducting adequately controlled experiments A proper review of the many complexities involved in adrenal neurohypophysial renal relations cannot be undertaken here reference is made to recent reviews by Gaunt *et al* (1957) Dingman (1958) and Relman and Schwartz (1958)

Potassium depletion Potassium depletion due to a variety of causes has been firmly established as a condition in which *concentrating* function may be markedly impaired in man and experimental animals (Relman and Schwartz 1956 1958 Hollander *et al* 1957b Mudge 1958) The tendency to isosthenuria cannot be overcome by injections of large doses of Pitressin The associated polydipsia may itself be a contributing factor in the sense shown by the data of Epstein *et al* (1957a) discussed previously Apparently a systematic study of the *diluting* process in potassium depletion has not been made in which C_m is varied during maximum sustained water diuresis to learn if there is an augmented C_{H_2O} similar to that induced by cortisone (Raisz *et al* 1957)

Age Very young and very old individuals of a number of species have been shown to have a more limited range of concentrating and diluting function Papers by Shock (1946) McCance (1950) Heller (1951) Barnett and Vesterdal (1953) Boyarsky and Smith (1957) and Dicker and Nunn (1958) should be consulted for details and additional references Proper assessment of such data requires that the role of *variations in GFR and* C_m *be taken into account*

Renal diseases Baldwin *et al* (1955) investigated the concentrating process in renal diseases and measured $T\,m_{H\,O}$/GFR for acute and chronic diseases In most patients some concentrating function was demonstrable despite fixation of specific gravity in some

Vasopressor Action

This long established pharmacological action of vasopressin has recently been shown to be of possible physiological interest as well Wagner and Braunwald (1956) found that in patients with autonomic

with data in Figure 2) These data are in accord with observations of West *et al* (1955) Jones and de Wardener (1956) and de Wardener and Herxheimer (1957) Epstein *et al* (1957a) however carried the comparison of the two regimens further by measuring maximum free water absorption ($T^cm_{H_2O}$) during osmotic diuresis by the methods of Zak *et al* (1954) This value averaged 6 7 ml (5 0 to 7 5) per minute in the postdehydration studies and was reduced by the forced hydra tion regimen to an average of 4 1 (2 5 to 4 8) per minute Some intra and/or extra renal conditioning or adaptation must be invoked to account for most of these changes

The attainment of highest osmotic U/P and lowest V in the true oliguria of dehydration therefore probably is the resultant of (1) slight to moderate decrease in GFR (2) reduction in C_m and (3) adaptation to chronic dehydration As a probable corollary secretion of less ADH and a lower plasma concentration would be required in this circumstance In this framework the kidney might be said to be more sensitive to ADH

Chronic overhydration on the contrary leads to a decrease in concentrating ability and an apparent decrease in sensitivity to ADH Kleeman *et al* (1958) have recently described such a case in which the severely impaired concentrating ability in a patient with psycho genic polydipsia was gradually restored toward normal when a normal fluid intake was imposed

A parallel study on the diluting process has not been made

Influence of level of protein in the previous diet on the concen trating process Epstein *et al* (1957b) systematically examined maxi mum osmotic U/P V and maximum free water reabsorption in normal subjects who ate high and low protein diets for 3 days They dis covered another well marked adaptation clearly independent of the adaptation to chronic under and overhydration just described Maxi mum osmotic U/P and $T^cm_{H_2O}$ were greater in those subjects on the high protein diet Unexpectedly — and most interestingly — daily sup plements of preformed urea to a low protein diet had the same effect on these parameters as did high protein feeding Acute admin istration of urea had no effect

A parallel study on the diluting process has not been done

Adrenal cortical hormones In human subjects chronically treated with cortisone or hydrocortisone Raisz *et al* (1957) found a somewhat higher C_{H_2O} and lower osmotic U/P during suppression of ADH secretion after administering standardized water loads even when

Normal men showed no decrease of blood pressure during 2 hour intravenous infusions of 10 or 20 units per hour Again as with vasopressin it is possible that endogenous oxytocin may normally produce subtle vascular effects which the autonomic reflexes tend to mask

Possibly related to this aspect of oxytocin activity are the interesting effects in dogs reported by Brooks and Pickford (1958) They found that oxytocin (synthetic) given alone as a single injection (5 0 milli units per kilogram) or as a constant infusion (0 2 milliunit per minute per kilogram) resulted in a marked increase in renal blood flow (diodone clearance) When oxytocin was combined with vasopressin in a ratio believed by Abrahams and Pickford (1954) to represent that in which the two substances are normally released endogenously (twenty to thirty times as much oxytocin as vasopressin) the renal blood flow remained unaffected The same doses of vasopressin alone had little effect on the renal blood flow Both hormones alone or in combination did not alter GFR significantly These authors postulate that vasopressin and oxytocin may have an important joint role in maintaining the normality of the renal vascular system A somewhat similar balancing action of the two hormones on the renal circulation of the rat was shown by Dicker and Heller (1946) Even more than this was claimed by Demunbrun *et al* (1954) In dogs with extensive neurohypophysial and hypothalamic lesions and polyuria Pitocin and purified oxytocin were able promptly to restore to normal not only the renal plasma flow but the depressed GFR and Tm_{PAH} (maximum tubular excretion of para amino hippurate) as well

NATURE OF THE CIRCULATING HORMONES

There seems to be general agreement that virtually all of the hormone in blood is confined to the plasma If this is so there are two main possibilities (1) that all of the vasopressin and oxytocin in the blood is present in the form of free octapeptides in solution in the plasma and (2) that all or most of the hormones are part of or attached to large molecules presumably protein in the plasma In the latter case two chief probabilities exist first that the bound form is analogous to the van Dyke protein isolated from beef posterior pituitary glands or second that the octapeptides are bound to plasma proteins Since the concentrations of the hormones in blood are extremely small there are no substantial data available which directly reveal the nature of the circulating form in *physiological* conditions

nervous system deficiency (postural hypotension hypohidrosis and impotence) intravenous injections or infusions of purified vasopressin or Pitressin in ranges of only 10 milliunits to 100 milliunits or 1 to 20 milliunits per minute respectively produced well marked increases in arterial pressure In normal subjects these doses had no pressor effect Prior injection of the ganglionic blocking drug tetraethylammonium chloride in normal subjects increased their sensitivity to the pressor action of vasopressin It seems likely that plasma concentrations of vasopressin associated with physiological antidiuresis may have subtle vascular effects which are normally obscured by autonomic reflexes

The vasopressor potency of oxytocin is only a small fraction of that of vasopressin (see Table I)

Oxytocic Action

The frequency and intensity of uterine contractions are increased by oxytocin and vasopressin The relative potencies of the two octapeptides depend on the method of oxytocic bioassay Two main functions have been ascribed to oxytocin in the regulation of uterine function (1) facilitation of sperm transport in the nonpregnant female and (2) facilitation of delivery of the fetus at the end of pregnancy For detailed discussion of this subject reference is made to the recent excellent reviews by Harris (1955) and Fitzpatrick (1957)

Milk Ejection

Milk ejection in the lactating female is the second most clearly established hormonal activity of the neurohypophysial principles The myoepithelial elements of the alveoli apparently lack motor nerves (Ely and Peterson 1941) and contract specifically in response to these hormones Many interesting facets of this problem beyond the scope of this chapter are discussed in the excellent reviews of Cowie and Folley (1957) and Harris (1955)

Avian Depressor Action

This activity is exhibited by oxytocin and by both vasopressins (see Table I) That this action may be of more than pharmacological interest is suggested by the observation of Wagner and Braunwald (1956) that intravenous injection of 1 unit of oxytocin lowered the blood pressure in 3 men with autonomic deficiencies to the range of 48 to 65 mm of mercury systolic and 34 to 45 mm of mercury diastolic

oxytocic and pressor activities of synthetic oxytocin and Pituitrin (Parke Davis and Co) added to isotonic saline or Krebs Eggleston phosphate saline buffer and to human plasma in cellophane bags dialyzed more slowly from the plasma than from the saline Inulin which is known not to be bound to plasma proteins disappeared from plasma and saline at the same rate when dialyzed under the same conditions The pressor bioassay method was not described but probably involved injection of human plasma into rats Nonspecific reactions might therefore have occurred which could alter the con clusions

However two important reports indicate that *endogenous* hormonal activity present in jugular venous blood in abnormally large con centrations following intracarotid or intracardiac injection of strongly hypertonic salt solutions exists mainly in the form of large molecules In the first of these the evidence is indirect Ames *et al* (1950) in fused 0 5 molar sodium chloride at the rate of 4 to 8 ml per minute for as long as 90 minutes into one carotid artery of dogs anesthetized with chloralose External jugular blood assayed by intravenous in jection in normal hydrated dogs contained about 100 microunits per milliliter Although the blood was not studied by other techniques these authors subjected the urine excreted during such intracarotid infusions to ultracentrifugation bioassays indicated that most of the antidiuretic activity sedimented as though part of a large molecule When such urine was boiled for 3 minutes without being acidified the antidiuretic activity no longer sedimented However just bringing the urine to a boil and immediately recooling did not affect sedi mentation of activity The activity could be destroyed by treatment with thioglycollate The possibility that small molecules of ADH attached themselves to inert large molecular substances of urine after the urine was formed in the kidney was to some extent ruled out

More recently Thorn and Silver (1957) studied the antidiuretic material found in the serum one minute after 0 5 ml of 2 5 per cent sodium chloride had been injected intracardially in ether anesthetized rats Upon subjecting such serum to electrophoresis on starch blocks (barbital buffer pH 8 6 ionic strength 0 1 8 to 9 volts per centimeter for 41 to 48 hours at 4 C) it was found that all of the detectable activity migrated with the β globulin fraction The active serum as well as extracts of the segments of the starch block after electro phoresis were concentrated by ultrafiltration through collodion membranes which had a pore size such that only proteins and aggre

Only a few observations suggest that the hormones exist mainly in the free octapeptide form in plasma the weight of still insufficient evidence favors the possibility that all or most of the activity is bound to larger molecules

In support of the first possibility this reviewer has data from two experiments in which purified vasopressin kindly supplied by Dr du Vigneaud was added to normal dog serum and plasma (5 or 10

TABLE II Bioassay of Dog Plasma Impermeate (P) and Ultrafiltrate (U) in a Conscious Female Dog During Maintained Hydration*

Mat ial j t d	E g us eatinl e l (GFR)		$\frac{C_{osm}}{GFR}$ 100†		U m†		$\frac{V}{GFR}$ 100		$\frac{C_{H_2O}}{GFR}$ 100	
	A‡	B§	A‡	B§	A‡	C	A‡	C'	A‡	C
	ml /min		ml /100 ml		mO m/L		ml /100 ml		ml /100 ml	
P	87	91	1 9	2 8	39	5	13 6	5 1	11 7	8 3
U	91	89	3 1	4 4	56	77	15 2	5 8	12 2	7 2
P	91	84	2 7	4 3	55	134	13 9	7 6	11 2	9 3
U	92	90	3 0	4 1	64	132	13 0	7 2	10 0	8 4
P	91	80	2 9	4 1	58	166	13 9	8 8	11 0	10 0
U	93	79	2 7	3 7	64	144	11 8	6 8	9 1	7 8
10 mU	94	67	2 4	3 2	59	127	11 1	6 3	8 8	7 2
Average P	90	85	2 5	3 7	51	118	13 8	7 2	11 3	9 2
A e ge U	92	86	2 9	4 1	61	118	13 3	6 6	10 4	7 8

From Lau 1953 E t nal jugul blood d wn th d y bef from th m d g (16 Kg) Th rginin as p es i ki dly ppll d by D du Vign ud was add d i 0 5 m llilit to 24 5 m llil t f th hep rin ed pl sma to nak final co t t f 10 m llunit p millilit Th mixtu e w ult af lte ed through V king ll phan und 100 mm f m ry p pplied by 6 pe t CO and 94 p t O_2 f 2 h ur t 4 C A co t l l t f th as p s i in toni al was p nd d i llophan bag with t p i 22 h t 4 C On millilite ject ns f imp n t (P) ult f lt t (U) and co trol sol ti (10 m ll units) w m d thro gh an i dw lling edl f leg i b twee the ighth and ni th m t f the p iod co po di g to C l mn A and washed i with 10 mill lit fi to i alin An i jecti as gi en ry 40 m t U we collected ev ry 10 mi t a in b ing used to t mi te th coll tions

† T tal l t in uri e was al ul ted th m f millim l nc tr ti ns f ea and eatinin plus twi the di m nd p ta ium nc t ti ns Plasma m l r nc trati was tak 280 milli mol p lit th oughou t.

‡ Col mn A A t al al during cc s i ontrol p i ds ear th d f wh h th i ject ns w gi

§ Column B A t al al es d ing the fir t po t i j ti p iod maxim m hanges oc u red thi p iod

Col m C D ff nc b tw th al in th fi t po ti jectio p i d mi th al i th spo ding trol pe i d.

milliunits per milliliter) (Lauson 1953) The serum was dialyzed against isotonic saline and the plasma was ultrafiltered both during 18 to 22 hours in the cold Upon injection intravenously into a conscious hydrated assay dog of the impermeate dialyzate and ultrafiltrate the changes in urine flow osmolar concentration (U_{osm}) and free water clearance suggested that relatively little of the vasopressin had been bound to proteins (Table II)

On the other hand Heller and Lederis (1957) found that the

some way to one or another normal plasma protein Thus the distinctions between rat serum endogenous ADH activity and the van Dyke protein are as stated by Thorn and Silver only tentative Their experiments provide the most advanced data relevant to the problem of the nature of the circulating neurohypophysial hormones and indirectly *of the nature of the hormones as secreted into the* blood Yet important questions remain For example even though an exact understanding of the nature of the circulating hormone obtained under unphysiological experimental conditions similar to those used by Ames *et al* (1950) and Thorn and Silver (1957) should be forthcoming it would still remain to be shown that ADH present in the blood exists in the same form under physiological conditions *

Dingman (1958) has made a beginning on the difficult problem of localizing *endogenous* ADH in human plasma protein fractions In one experiment Cohn s Fraction II + III showed apparent antidiuretic activity when assayed in a *dog* with diabetes insipidus Unfortunately the data on free water clearance (C_{H_2O}) and urinary osmolar concentration (U_{osm}) were variable throughout making it difficult to evaluate the adequacy of the assay The activity apparently localized in this fraction was thought to account for a concentration of about 5 microunits per milliliter in the original plasma That nonspecific (probably vascular) effects might have resulted from injection of human plasma protein concentrates into dogs seems a strong possibility inasmuch as urine flow ceased altogether after injection of the larger dose of Fraction II + III

Additional indirect evidence that circulating ADH may be in large molecular form under more physiological conditions is found in *several other studies on the dialyzability of endogenous urinary anti*diuretic material Gilman and Goodman (1937) found that most of the antidiuretic substance in the urine of rats dehydrated 24 to 72 hours did not pass through the cellophane dialyzing membrane during dialysis against tap water Ames and van Dyke (1950) found nondialyzable ADH like material in urine of normal kangaroo rats Although others have found that endogenous antidiuretic material

* *Note added in proof In recent experiments Lauson and Bocanegra (1959)* have found that the naturally secreted canine ADH contained in the plasma of external jugular blood removed during the last half of a fatal hemorrhage is roughly 80 per cent ultrafiltrable through cellophane at 4 C under 90 mm of mercury pressure (5 per cent CO_2 95 per cent O_2 saturated with water vapor) Since arginine vasopressin is probably the active octapeptide in both dog and rat these data and those of Thorn and Silver (1957) suggest a large species difference in the degree of binding of ADH by plasma proteins

gates with molecular weight of 70 000 or larger were retained the ADH activity was all retained by the collodion The authors pointed out that if the activity were in the form of the van Dyke protein (van Dyke *et al* 1942) with a molecular weight of only 30 000 (it was apparently implied that the hypothetical rat analogue of the bovine van Dyke protein would also be a protein of molecular weight less than 70 000) it should have passed through the filter to an appreciable extent Dialysis of the active serum in cellophane against four changes of 0 2 normal acetic acid resulted in complete displacement of the activity into the acid dialyzate Dialysis against 20 volumes of water resulted in loss of part of the activity In this last respect the active material seems to differ somewhat from the bovine van Dyke protein (*Acher and Fromageot 1955*)

Thorn and Silver (1957) also found that the antidiuretic material in the rat serum showed patterns of inactivation by proteolytic en zymes identical to those shown by purified vasopressin Pitressin and the van Dyke protein Like these other substances the rat serum antidiuretic material was inactivated by thioglycollate

Subsequently Thorn (1958b) reported that when highly purified vasopressin or synthetic oxytocin was added *in vitro* to normal rat serum and then subjected to electrophoresis at 4 C the activity moved in a fairly broad zone with the globulin fractions not just with the beta fraction as found for endogenous hormone

Several comments may be made First there can be little doubt that the endogenous serum activity studied by Thorn and Silver was truly of neurohypophysial origin Second there are only two points of difference between their material and the van Dyke protein (1) the slow passage of the serum activity through cellophane when dia lyzed against water and (2) the retention of the serum factor by collodion which had pores large enough to permit passage of a substance of molecular weight of 30 000 Inasmuch as the rat equiva lent of van Dyke s bovine posterior pituitary protein has not yet been isolated it is possible that it might turn out to have a molecular weight in the range of 70 000 or more and if so it could have been retained by Thorn s collodion However the only other known van Dyke protein that isolated from hog pituitary glands and studied by Acher (1958) also has a molecular weight of about 30 000 Since van Dyke s protein apparently has never experimentally been dissolved *in bovine plasma* and then studied it is conceivable that such posterior pituitary proteins themselves might become bound in

nature of the stimulus Van Dyke *et al* (1957) have briefly summarized the present status of this problem and observed that apparently no satisfactory experiments in which vasopressin and oxytocin have been simultaneously measured in blood plasma or serum have been reported as a basis for deciding what amounts of the two hormones are liberated simultaneously The concept of simultaneous liberation rests therefore on indirect evidence

Abrahams and Pickford (1954) made simultaneous observations on urine flow and uterine movements in dogs during water diuresis Application of osmotic chemical and emotional stimuli always invoked simultaneous antidiuresis and increased uterine motility To match these effects by single intravenous injections of Pitressin and Pitocin it was necessary to inject intravenously less than 5 milliunits of Pitressin and fifteen to thirty times as many milliunits of Pitocin After surgical induction of diabetes insipidus polyuria was associated with decrease or absence of spontaneous uterine activity Holland *et al* (1958) recently reported that intracarotid injection of hypertonic saline induced milk-ejection in lactating rabbits Thus stimuli usually associated with release of ADH seem also to cause release of oxytocin in amounts many times larger

Conversely the stimulus of *suckling* would be expected on teleo logic grounds to release only oxytocin Nevertheless submaximal antidiuresis has been observed shortly after application of the suckling or milking stimulus to water loaded lactating rabbits (Cross 1951) cows (Peeters and Coussens 1950) dogs (Kalliala *et al* 1952) and women (Kalliala and Karvonen 1951) These data suggest liberation of relatively small amounts of vasopressin in association with rela tively large amounts of oxytocin

Release of oxytocin during *coitus* is suggested by the observations (1) that in several species uterine activity increases facilitating it is believed sperm transport (see review by Fitzpatrick 1957) and (2) that in lactating women milk-ejection may occur (Harris and Pickles 1953) The fact that antidiuresis also may occur (Friberg 1953) suggests dual release again with oxytocin predominant.

Andersson (1957) observed a constant association of antidiuresis and milk-ejection during electrical stimulation of the supraoptic nuclei or of the supraoptic neurohypophysial tract in lactating goats Harris (1955) has also reviewed results of electrical stimulation which are in accord with the dual release concept. However Thorn (1958a) has pointed out that results of such stimulation are inconclusive because

dialyzes from urine we are inclined to ascribe *more validity to the* observations showing nondialyzability

ENTRY INTO THE BLOOD

Formation

The present view originally set forth by Scharrer and Scharrer states that the hormones are formed in the neurons of the supra optic and paraventricular nuclei of the hypothalamus and migrate in or along the axons to be stored in the posterior lobe of the pituitary gland (see recent reviews by the Scharrers 1954a b Barg mann 1957 Harris 1955) Liberation into the blood is assumed to be under the control of the hypothalamic neurons which when appropriately stimulated release the hormones from their endings in the posterior lobe into the capillaries The ultrastructure of these endings and capillaries seems to be well adapted for this function (Palay 1955) It is interesting that Scharrer and Scharrer (1954a) Rothballer (1953) and Hanstrom (1952) have seen neurosecretory granules identical with those associated with axons in the neuro hypophysis and presumed to contain hormones in the capillaries of the posterior lobe of the dog rat and camel respectively under con ditions which favor release of large amounts of ADH The amounts of vasopressin and oxytocin and the *V/O* ratios contained in the sub divisions of the neurohypophysial system of several species vary considerably (van Dyke *et al* 1955 1957) A *V/O* ratio of less than 1 0 has never been found in the posterior lobe when adequate assay methods were used Approximate *V/O* ratios in the posterior lobe *were tabulated by van Dyke et al (1957) for the dog camel rat ox* hog monkey and man they were 1 5 3 3 1 3 1 4 1 1 1 5 and 1 2 respectively

Liberation

The simultaneous release concept Inasmuch as knowledge of the content of the hormones in the neurohypophysis can give no real information on rates of liberation into the blood it is more useful to turn now to the physiologically important question of whether vasopressin and oxytocin can be separately released in response to appropriate stimuli or whether they are always released together in a constant or inconstant proportion regardless of the

nature of the stimulus Van Dyke *et al* (1957) have briefly summarized the present status of this problem and observed that apparently no satisfactory experiments in which vasopressin and oxytocin have been simultaneously measured in blood plasma or serum have been reported as a basis for deciding what amounts of the two hormones are liberated simultaneously The concept of simultaneous liberation rests therefore on indirect evidence

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even if separate fibers exist in the [supraoptic hypophysial] tract for the release of such hormones both kinds would probably react to such a stimulation

In brief the concept of simultaneous liberation has fairly convincing indirect evidence in its favor However the full meaning of this phenomenon if true has so many ramifications that they cannot be profitably speculated on at this time Direct assays by adequate methods of the venous blood draining the secreting region are certainly required

Natural Stimuli for Release of Antidiuretic Hormone

The dominant stimulus in physiological antidiuresis is the increase in effective osmolar concentration of the plasma resulting from progressive *loss of free water from the body as time elapses since a last ingestion* of water Verney s experiments (1947) led him to postulate the existence of osmoreceptors possibly located in the hypothalamus which are increasingly excited by a progressive increase in plasma osmolarity (see also O Connor 1950) The hypothetical osmoreceptors behave like slowly adapting neural receptors Their graded sustained excitation apparently results in graded sustained liberation of antidiuretic hormone and consequently graded sustained antidiuresis On the other hand sudden relatively large increases in plasma osmolarity resulting from injection of hypertonic solutions into the carotid artery or peripheral vein cause the rapid release of a slug of ADH (and oxytocin) which immediately reduces urine flow (Verney 1947)

Less clearly understood is the physiological role of the so called volume receptors in the antidiuretic system Smith (1957) drawing on a large and often confusing literature has written a challenging exercise in physiologic apologetics in which he tentatively accepts the hypothesis of Henry *et al* (1956) that receptors in the left atrium and intrapericardial pulmonary veins are stimulated by distention of these structures and that the resulting afferent impulses conducted in the vagi are inhibitory to the antidiuretic system This hypothesis together with its companion version concerning an antinatriuretic system seems to accommodate successfully a large number of stubborn facts which had resisted integration for some years In this view the antidiuretic system is subject to graded excitation or inhibition independently of changes in plasma osmolarity by way of the atrial receptors by neural impulses associated with pain emotion suckling

or coitus or by the action of various chemical agents (see also reviews of Pickford 1952 Harris 1955 Thorn 1958a)

Dingman (1958) has proposed the interesting hypothesis that the release mechanism responsive to osmotic stimulation possibly located in the posterior lobe (Dingman *et al* 1957) is separate from another release mechanism located in the hypothalamus and responsive to neural influences (as exemplified by nicotine injection) Smith (1957) has the various factors acting on a single final release mechanism The thoughtful and detailed reviews of the volume regulation prob lem by Heller (1955) and Grossman (1957) should also be consulted

NORMAL RATES OF LIBERATION OF VASOPRESSIN AND OXYTOCIN

Several investigators have estimated normal rates of liberation by suppressing endogenous hormone secretion and then mimicking normal physiological effects on target organs by infusing intravenously to equilibrium or by injecting exogenous extracts or more recently pure octapeptides Constant infusions in subjects given sufficiently large maintained water loads are a valid means especially for estimating ADH liberation because normally this hormone is liberated continu ously at a nearly constant or perhaps slowly increasing rate whenever the individual goes several hours without drinking Use of the single intravenous injection method is also reasonable because sudden libera tions are probably common occurrences This method is particularly appropriate in relation to estimation of the quantity of oxytocin released prior to milk ejection

Constant Infusion of Antidiuretic Hormone

Studies of this kind were first done by Shannon (1942b) whose work with normal dogs and dogs with experimental diabetes insipidus has served as a model for all subsequent investigators Hare *et al* (1943) estimated the rates of liberation prevailing during rapid infusion of hypertonic saline in normal dogs Verney s (1947) experiments in which exogenous pituitary extracts were infused intravenously for 40 minutes led to estimates of the physiological rates of ADH libera tion A study on normal dogs subjected to varying degrees of super imposed solute diuresis was recently reported by Orloff *et al* (1958) For man two reports are available In the first by Lauson (1951) a

series of observations were made on 1 normal subject and in the second by Hollander *et al* (1957a) studies on 8 normal men are detailed Dicker s (1954 1957) seem to be the only papers on the rat reporting results with this method Table III presents data selected

TABLE III Antidiuretic Responses Achieved at Equilibrium During Constant Intravenous Infusions of Vasopressin

Speci s and ubject	Body w ight	S f ar	Inf sio t		Estim ted C m/GFR	E t m ted d gr f antid ur l †	A th
	Kg	M 2	μ U/mi /Kg	μ U/min./M.2	ml /100 ml		
Man							
H. L	74	1 84	0 9	34	1 8		Lau (1951)
H. L.	74	1 84	2 9	111	1 3	—	Lauson (1951)
R S	76	1 93	0 2	9	2 0		Hollander et al
E D	76	1 92	1 6	63	3 0		(1957)
E D	76	1 92	3 4	134	7	++ to +	Hollander et al
T C	83	2 1	2 1	85	1 2	to ++	(1957a)
Dog							
D‡	8 8	0 43	1 9	39	low	+	Shannon (1942b)
D	8 8	0 43	9 5	194	low	—	Shannon (1942b)
E	14 1	0 59	5 9	141	5 4		Shannon (194 b)
F	14 1	0 9	23	565	4 8		Sha no (194 b)
Nicky	17	0 67	4 0	90	low	to ++	Verney (1947)
Se eral	16 20	—	8 3	—	1 0		Orloff tal (1958)
Several	16 20	—	16 7	—	1 0		O loff tal (1958)
Several	16 20	—	25	—	1 0	to ++++	Orloff et al (1958)
T	19	0 72	25	660	2 2		O loff et al (1958)
T	19	0 72	25	660	6 7		O loff tal (1958)
T	19	0 72	108	2850	7 3	—	O l ff et al (1958)
S§	12	0 53	250	5650	20	—	Hare et al (1943)
R t							
No mals	0 25	0 036	50	350	p obably high	0 to	Di ker (1954)
Norm l	0 25	0 036	100	700	10		Dicker (1957)
Normals	0 25	0 036	200	1400	10	—	Di ker (1957)

Shanno used Pituit all th used Pitr i O loff et al used purifi d as p in m of th ex p im ts

† Value f ++ to +++ ++++ as ign d to i di te with nsid abl rtai ty th t early o just maximal antidi i was ttai ed. Wh th l is unde lined it mean th t g te pons esult d fr m high inf i at De ignati ns f ++ ++ ry arb t ry by c mp n

‡ Dog with exp ime tal diabet insipid W ter w all d ad l b until b ginni g f infu i th as i all the w te l ad f 1 5 to 2 p ent f body weight (man) o 5 p t (dog and t) wa mai tained du ing th infusi n.

§ Dog with diab te in ipidu W ght f dog was not gi but w an ughly estimat it to h b 12 kg becau the cont l GFR was bo t 40 ml p min te W t was all d d lib until beginning f inf i which cons ted f 2 5 pe ent sodi m chlorid gi en t th t f 10 ml, p mi t f 45 mi te th n dic ted P't es in w incl ded i thi all infusio GFR i ased by abo t 50 pe t by th end f th infusi Se Laus (1951) fo d tailed di cu i f th exp iment

from these sources to indicate the approximate ranges of the estimated physiological rates of ADH secretion in the three species man dog and rat

It must be emphasized that it is sometimes very difficult to decide whether the antidiuresis achieved during a given rate of infusion

is still slightly submaximal or is already supramaximal (and therefore associated with plasma hormone concentrations which may be just adequate for the maximum effect under the existing conditions of GFR and C_{osm} or may be indeterminately higher) Furthermore it has been clear since the work of Shannon (1942b) and of Hare *et al* (1943) (for a critical review of these important papers see Lauson 1951) that higher rates of infusion are required to achieve maximum antidiuresis when the rate of solute excretion or C_{osm} moderately or greatly exceeds the usual normal range The data of Orloff *et al* (1958) show this most clearly

Thus in a strict sense there is no normal range of rates of ADH liberation but rather one in which depending on C_{osm} GFR and other influences detailed in a previous section (p 237) the threshold and upper limit are variable In selecting and evaluating the data in Table III these limitations have been taken into account especially in making the semi quantitative estimates of degree of antidiuresis relative to the GFR and C_{osm} (when known)

Data on Human Beings A measurement of great value in deciding whether a given infusion rate is just submaximal or is significantly supramaximal is the time during which the full antidiuretic effect persists after the infusion is stopped Figure 3 constructed from the postinfusion data of four of Lauson s experiments (1951) on a single human subject indicates that for a C_{osm} range of about 1 to 2 per cent of GFR infusion of 12 milliunits per hour (2 9 microunits per minute per kilogram) for 2 hours resulted in something near to just maximal antidiuresis 42 milliunits per hour (10 microunits per minute per kilogram) was clearly supramaximal The rate of 10 7 milliunits per hour (2 1 microunits per minute per kilogram) in Subject T C of Hollander *et al* (1957a) was probably not quite maximal [osmotic U/P rose to 2 2 and C_{H_2O} fell to -0 8 ml per minute per 1 73 M^2 for C_{osm} of only 1 6 ml per minute per 1 73 M^2 (see Fig 2)] *In Subject E D of Hollander et al (1957a)* C_{osm} had been increased to over 7 ml per minute per 1 73 M^2 by a previous diet high in protein and salt yet an infusion rate of only 14 milliunits per hour per 1 73 M^2 (3 4 microunits per minute per kilogram) resulted in a nearly maximum antidiuresis at equilibrium as judged from the attained osmotic U/P ratio of 2 1 and C_{H_2O} of -3 6 ml per minute per 1 73 M^2 (see Fig 2) This suggests that the apparent sensitivity to ADH in this subject was not much less than in T C whose C_{osm} was considerably lower

It seems probable therefore that for the osmolar clearance range of 1 to 2 ml per minute per 1 73 M 2 in normal *hydrated* men whose previous nutrition and fluid intakes are ordinary maximum anti diuresis is attained with infusion rates somewhat greater than 12 milliunits per hour per 1 73 M 2 (greater than 3 microunits per minute per kilogram) and definitely less than 42 milliunits per hour per 1 73 M 2 (less than 10 microunits per minute per kilogram)

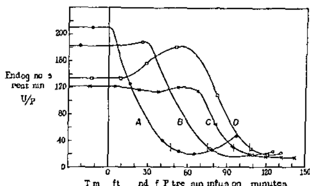


FIGURE 3 Changes in the urine/plasma concentration ratio of endogenous creatinine *U/P* following abrupt cessation of 2 hour infusions of Pitressin at four rates on different days in the same hydrated normal man (74 kg 1 84 M 2) This ratio varies inversely with the urine flow Values for each urine collection period were plotted at the midpoint of the periods and the smooth connecting curves were drawn by eye *Curve A* 12 3 mU/hr (2 9 μU/min /kg) *curve B* 42 mU/hr (9 5 μU/min /kg) *curve C* 190 mU/hr (43 μU/min /kg) *curve D* 245 mU/hr (55 μU/min /kg) The variation in maximum *U/P* obtaining near the end of each Pitressin infusion reflects differences in concurrent osmolar clearance antidiuresis was maximal in each instance The increase of *U/P* in *curve D* indicates a decreasing osmolar clearance while antidiuresis was still maximal (From data of Lauson 1951)

Data on Dogs In Shannon s (1942b) four dogs with diabetes insipidus infusions of Pituitrin were begun without preliminary loading with water such as is necessary when normal dogs are employed Because large water loads usually increase GFR (Shannon 1942a b) the level of GFR in the dogs with diabetes insipidus which were merely allowed water ad libitum was relatively lower (C_m was also probably lower) than in heavily hydrated normal dogs In this sense dogs with diabetes insipidus hydrated ad libitum are more appropriate subjects for estimating the normal rates of ADH liberation Shannon observed graded antidiuresis consistently only in the range of up to 5 milliunits per hour Apparently maximum antidiuresis was attained with rates of 5 milliunits per hour in three of the four dogs and at 10 milliunits per hour in the fourth (approximately 7 to 15 micro

units per minute per kilogram) When water loading with its resultant increase in GFR and C_m was superimposed higher rates of hormone infusion seem to have been required to achieve maximum antidiuresis both in the dogs with diabetes insipidus and in normal dogs (Shannon 1942b) The data from Verney s (1947) experiments which utilized a very different method of study (intracarotid infusion of hyperosmolar solutions) are in good agreement with those of Shannon s experiments on dogs with diabetes insipidus

The study of Orloff *et al* (1958) represents the most complete and best controlled effort so far to estimate normal rates of ADH liberation All of the observations were made in repeated studies on a few normal dogs maintained on a relatively constant high protein diet during each experiment standardized hydration was maintained These investigators first established the upper (no ADH administered) and lower (maximum ADH effect resulting from infusion of 50 milliunits per hour per kilogram) limits of urine flow over a wide range of C_m achieved by superimposing osmotic diuresis on the water diuresis Next they infused arginine vasopressin (du Vigneaud s purified product) at one of three submaximal rates until a steady state supervened at lower and again at higher rates of osmolar clearance The results are shown in Figure 4 It is evident that a given infusion rate is less able to reduce free water clearance at higher than at lower rates of osmolar clearance The same infusion rate (1 5 or 4 0 milliunits per hour per kilogram) in the same animal on the same day produced hypertonic urines when C_m was in the range of 0 8 to 2 4 ml per minute and hypotonic urines when C_m was increased into the range of 7 to 12 ml per minute It can be concluded that under the conditions of their observations an infusion rate of 1 5 milliunits per hour per kilogram (25 microunits per minute per kilogram) is nearly adequate to promote maximal antidiuresis only when C_m is fairly low

Data on Rats Dicker (1954) infused Pitressin intravenously at constant rates for 30 minutes into hydrated rats under ethanol anesthesia For water loads up to 8 per cent of body weight rates of 160 to 200 microunits per minute per kilogram were required for maximum antidiuresis In rats maintained on water loads of 9 to 15 per cent no antidiuresis resulted from infusion rates of less than 200 microunits per minute per kilogram and effects of higher rates were variable It seems clear that such water loads are excessive and the apparent insensitivity can probably be attributed to increase in GFR and C_m Conscious rats hydrated to 5 per cent of body weight

showed moderate and maximum antidiuresis (hypertonic urine) with infusion of 100 and 200 microunits per minute per kilogram of Pitressin (Dicker 1957) C_{osm} was about 0 3 ml per minute per kilogram or approximately 10 per cent of GFR (Dicker and Heller 1946) This is comparable to a C_{osm} of about 10 or 12 ml per minute in a

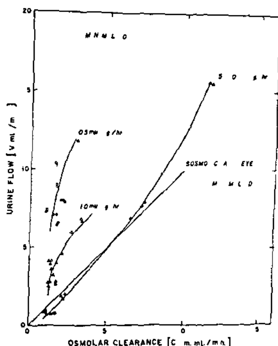


FIGURE 4 Urine flow as a function of osmolar clearance in normal hydrated dogs Individual data are from 3 dogs given constant infusions of vasopressin (0 5 1 0 and 1 5 mU/hr /kg) long enough to achieve equilibrium before the urine collections were begun The dashed lines indicate the upper and lower limits of urine flow during suppression of ADH secretion (maximal water diuresis) and during infusion of Pitressin at supramaximal rates respectively (From Orloff *et al* 1958)

normal man and must be characterized as moderately high Presumably a rat would require less hormone to achieve maximum antidiuresis if C_{osm} were in the range of only 1 or 2 per cent of GFR (provided of course that complete suppression of endogenous ADH release could be achieved under this condition) However as is discussed in a later section (p 259) the rate of removal of ADH from the plasma is relatively much higher in the rat than in dog or man Higher secretory rates would be demanded in the rat if the physiologically effective plasma concentrations were roughly comparable in the three species

Constant Infusion of Oxytocic Hormone

A remarkable study was reported by Caldeyro Barcia and associates (1957) in which a series of graduated constant infusions of synthetic oxytocin (Syntocinon, Sandoz) were given intravenously to women in late pregnancy (36 to 40 weeks) prior to the onset of labor. The infusion was started at the rate of 1 milliunit per minute and continued for 1 hour; then without interruption it was doubled every hour and usually continued through a rate of 32 milliunits per minute. Resulting uterine activity was assessed by a method combining frequency and amplitude of amniotic fluid pressure waves and expressed as "Montevideo units." Equilibrium was probably very nearly reached by the end of each 1 hour infusion. Their data relating the increase of uterine activity (ΔUA) to the infusion rate (inf) fitted the equation

$$\Delta UA = \Delta_{max} UA \; (1 - e^{-R \cdot inf})$$

where $\Delta_{max} UA$ is the hypothetical maximum increase and R is a coefficient characterizing the reactivity of the normal full-term uterus to oxytocin. The data were remarkably homogeneous. The value for $\Delta_{m} \; UA$ was 237 "Montevideo" units and for R was 0.21 when the infusion rate was expressed in milliunits per minute. In terms of "Montevideo" units, the average spontaneous uterine activity near term is 64 units; at the beginning of spontaneous labor it is about 100 units; at the *end* of the *first stage* of labor it is about 200 units; and during the *second* stage it is about 250 units. These activities are reproduced in women not yet in labor by infusions of 1, 8 and 16 milliunits per minute, respectively. It may be noted that, taking an ADH infusion rate of 6 microunits per minute per kilogram (0.42 milliunits per minute for a 70 kg man) as adequate for maximal antidiuresis under ordinary conditions (Table III), these rates of oxytocin infusion are about two and a half, twenty and forty times as great, respectively.

Single Intravenous Injections of Antidiuretic Hormone

Rapidity of ADH Release and Onset and Persistence of its Renal Effect. Verney (1947) applied electrical stimulation for 30 seconds to the lower abdomen or injected hypertonic solutions during 30 seconds or less into the carotid artery of dogs in water diuresis. The time courses of the resulting antidiureses were then carefully matched

by *single* intravenous injections of posterior pituitary extracts Thus it was possible to estimate the amount of endogenous ADH that the stimulus had caused to be released Verney s Figure 1 shows that *urine flow* (measured at 2 minute intervals) began to decrease within 2 to 4 minutes after the injection or electrical stimulation and that it reached a minimum within 8 to 10 minutes (Dicker 1953 has *shown similar rapidity of response to injection of* Pitressin in rats) If the antidiuresis was submaximal urine flow often began to increase again as soon as 2 minutes after the minimum value had been reached These *observations indicate (1) rapid acute release of ADH in* response to appropriate acute stimuli (2) virtually immediate response by the kidney and (3) a short persistence of the effect (i e very little lag *between the time of change in plasma concentration and the change* in urinary response) The latter point makes it valid to attempt estimation of the plasma concentration as a function of time from observed changes in renal response following acute endogenous ADH release or single intravenous injections This aspect is developed in a later section (see p 267)

Gradation of Response to Single Injections Antidiuretic responses can be graded as a function of dosage in the lower dosage range in terms of decrease in urine flow (or related parameters) until a maximum decrease is attained Further increase in dosage results in increase in duration of the maximum effect The latter makes reasonable the assumption that when a maximum antidiuresis persists for more than a few moments the plasma concentration of ADH initially has been higher than the minimum required for maximal antidiuresis

Estimation of Normal Concentrations of ADH in Plasma The degree of antidiuresis resulting transiently from a single intravenous injection of vasopressin has long afforded a crude measure of physiological concentrations of ADH in the plasma Theobald (1934) noting that intravenous injection of 5 to 10 milliunits of Pituitrin in hydrated *human subjects resulted in approximately maximum antidiuresis* lasting a few minutes calculated that this response corresponded to a transient concentration in the arterial blood of about 2 microunits per milliliter (or roughly 4 microunits per milliliter of plasma) This concentration must first decrease before any degree of water diuresis can occur in a subject experiencing maximum antidiuresis Theobald s estimate of normal ADH concentrations in plasma has survived numerous challenges up to the present day

Estimation of the Rate of Removal of ADH from the Plasma In

the water loaded subject the time course of change in urine flow (or other parameters) is a crude but useful measure of the rate at which ADH is removed from the blood The fact that diuresis returns to control values following a single injection of a submaximal dose of vasopressin in about 20 to 30 minutes in the rat 40 to 50 minutes in the dog and 50 to 70 minutes in man immediately affirms that the fraction of the volume of vasopressin distribution which is cleared per minute is greatest in the rat and least in man This point will be developed more fully in a later section

Single Intravenous Injections of Oxytocin

Estimates of the quantity of oxytocin released in response to the natural suckling stimulus may be made by determining the intravenous dose of oxytocin required for milk ejection For example in the lactating rabbit Cross and van Dyke (1953) found that 1 milliunit of purified oxytocin (du Vigneaud) usually evoked a threshold response and that 50 to 100 milliunits were required for a maximum response Purified vasopressin (du Vigneaud) had to be given in doses averaging 6 1 times as large in terms of standard posterior powder to achieve comparable responses (confirming observations of Turner and Cooper 1941) The corresponding range of estimated oxytocin release in lactating rats is from less than 20 to about 100 milliunits per kilogram (Grosvenor and Turner 1957) In lactating swine Whittlestone (1952) showed that a dose of 500 milliunits of purified oxytocin given intravenously was clearly suprathreshold purified vasopressin had about one fifth the milk ejection potency of oxytocin In lactating women early in the post partum period Nickerson *et al* (1954) found that 500 milliunits of purified or synthetic oxytocin given intravenously consistently evoked a minimal milk-ejection response Recently Beller *et al* (1958) found that sensitivity of lactating women to oxytocin increased progressively after the second post partum week Threshold intravenous dosage for milk-ejection in most of their patients was 10 milliunits or less after the second week Very large doses (3 to 5 units) inhibited milk ejection

Similarly uterine sensitivity to oxytocin has frequently been estimated in terms of the intravenous dose required to induce contractions Fitzpatrick (1957) has tabulated data from the literature on the *minimal* doses required to stimulate the highly sensitive uterus of pregnant women near and at term From these data he estimated that transient plasma concentrations of from 1 5 to 30 microunits per

milliliter are sufficient to cause a minimal response Therefore the quantities of oxytocin needed to be liberated into the blood to elicit *minimal* physiological responses in the uterus during parturition are apparently not very much greater than the quantities of vasopressin required for physiological antidiuresis For further discussion of the effects of oxytocin on the uterus the reader is referred to reviews by Harris (1955) and Fitzpatrick (1957)

SITES OF REMOVAL OF THE HORMONES FROM THE PLASMA

The concentration of these hormones in arterial plasma (it is assumed that they neither become bound to nor permeate into erythrocytes) at any moment is a function of the rate of secretion into and the rate of removal from the plasma The concentration in any vein is influenced in addition by the rate of removal (or rate of secretion as in the case of veins draining the hypothalamic region) in the specific organ or tissues drained by the vein in question The rate of removal in a particular organ or tissue is given by the product of the blood flow and the difference of concentration between arterial blood and the venous blood draining the organ The total rate of removal is the sum of all regional removal rates The total rate of removal (microunits per minute) divided by the concurrent arterial plasma concentration (microunits per milliliter) is the total plasma clearance i e the virtual volume of plasma from which all hormone is removed per minute

Inactivation of ADH by the Blood

The first tissue to consider as a site of removal is the blood itself Pitressin added to heparinized blood and incubated 30 minutes or more at 38°C is not inactivated by the blood of normal rats (Heller and Zaidi 1957) and of normal human subjects (Dieckmann *et al* 1950) However the blood of pregnant women apparently contains an enzyme capable of inactivating Pitressin (Dieckmann *et al*, 1950 Croxatto *et al* 1953)

Removal of ADH by Liver and Kidneys in Rats

Ginsburg and Heller (1953b) injected 1 000 000 microunits per kilogram of Pitressin intravenously into rats When the renal circulation was occluded the slope of the curve of disappearance from plasma (plasma concentrations were measured by bioassay) was de

creased by more than one half The slope was also decreased by almost one half in rats in which hepatic blood flow was reduced to 30 per cent of normal by ligation of the celiac and mesenteric arteries With both renal and these splanchnic ligations the slope was only about 10 per cent of the normal Calculations made by the authors revealed the following striking conclusions (1) In intact rats the clearance of Pitressin (when injected in these very large doses) was equivalent to 81 per cent of the plasma volume per minute (2) the half life was only 51 seconds (3) the kidneys must have removed practically all the hormone from the blood which passed through them and (4) extraction of the hormone from the blood flowing through the splanchnic vascular bed (presumed to be by the liver and later found by Ginsburg 1957 not to involve the intestines) must have been almost as complete as in the kidneys The analogous experiments by Ginsburg (1954) on adrenalectomized rats gave results consistent with these Furthermore Ginsburg and Heller (1953a) showed that the concentration of *endogenous* ADH liberated under the combined stimulus of ether anesthesia and hemorrhage in venous blood representing mainly a mixture of blood draining liver and kidneys was considerably lower than in arterial blood and much lower than in external jugular blood

Dicker (1954) however reported data on the intravenous infusion of Pitressin in nephrectomized rats which are actually somewhat at variance with those of Ginsburg and Heller (1953b) although the author interpreted them as being confirmatory and Heller (1957) accepted the confirmation Four nephrectomized rats under ethanol anesthesia were given 100 to 300 microunits per minute per kilogram of Pitressin intravenously for 30 minutes Two to three minutes after cessation of the infusion blood was removed for bioassay of plasma ADH content The concentrations were 56 70 84 and 134 microunits per milliliter respectively Using the equations and assumptions of Ginsburg and Heller (1953b) which are discussed later (see p 272) the reviewer calculated that the average clearance of Pitressin in the absence of the kidneys was only about 5 to 10 per cent of the plasma volume per minute In the nephrectomized rats of Ginsburg and Heller however the clearance averaged 35 per cent of the plasma volume per minute Thus Dicker s data suggest that in normal rats the renal clearance represents around 90 per cent of the total clearance whereas Ginsburg and Heller s data attribute only 57 per cent of the total Pitressin clearance to removal by the kidneys

The data of Crawford and Pinkham (1954) indicate that the kidneys of the rat play an important part in the clearance of Pitressin

Removal of ADH by Liver in Dogs

Independently Mathe and Altman (1954) and Lauson (1953) sought to confirm in dogs the role of the liver in ADH removal under more physiological conditions During a prior operation a polyethylene catheter was tied into a splenic vein filled with heparin sealed and delivered through the incision On later days the unanesthetized dog was brought into maintained water diuresis and Pitressin in total doses of 0 5 1 5 25 or 250 milliunits (approximately 3 to 5000 micro units per minute per kilogram) was infused during periods of 5 or 15 minutes by the French investigators and in doses of 0 1 to 0 5 milli unit per minute (about 5 to 25 microunits per minute per kilogram) for 5 10 20 or 40 minutes by Lauson The antidiuretic effects (change in urine flow conductivity and osmolarity) resulting from an infusion via the splenic vein catheter were compared to the effects resulting from the same infusion administered before or after by way of one of the leg veins The quantitative differences in results for the two routes of administration were small and not statistically significant in the experiments of Mathe and Altman The data of Lauson also showed small questionable differences in some and no difference in other experiments Nevertheless it is possible that the liver of the dog does extract a small percentage of Pitressin from the blood flowing through it *

To confirm the results of Ginsburg and Heller (1953b) by another approach Ginsburg (1957) measured arterial blood pressure after single injections of Pitressin in doses of 40 to 80 milliunits per kilo gram into *rats* anesthetized with urethane and given Dibenzyline as an adrenergic blocking agent *After portal vein injection the blood* pressure rose only about half as much as after injection of the same doses into a peripheral vein Bocanegra and Lauson (1958) repeated these experiments in *dogs* under urethane and Dibenzyline portal and peripheral venous injections of equal doses of Pitressin resulted in essentially equal blood pressure responses

The data of Mathe and Altman and of Lauson on the dog seem to

* Note added in proof Using the same techniques Lauson and Bocanegra (1959) have recently begun a careful reinvestigation of this problem but this time with the knowledge that only small hepatic extractions could be expected In a series of experiments on the one dog studied to date the liver removed roughly 10 to 15 per cent of the ADH carried to it in the portal vein Therefore it seems possible that one third to one half of the total ADH clearance in the dog could be accounted

be at marked variance with those of Ginsburg and Heller (1953a b) on the rat As will be brought out later the discrepancy may be more apparent than real

TABLE IV Removal of Antidiuretic Hormone by Kidney*

Vasopressin infusion	Exogenous creatinine clearance (GFR)		$\frac{C_{osm}}{GFR} \times 100$†		U_{osm}†		$\frac{V}{GFR} \times 100$		$\frac{C_{H_2O}}{GFR} \times 100$		$\frac{U_{PAH}V}{GFR}$ (L/R)	
	A‡	B§	A‡	B§	A‡	C‖	A‡	C‖	A‡	C‖	A‡	B§
	ml./min.		ml./100 ml		mOsm/L.		ml./100 ml		ml./100 ml.			
PV	48	47	2.1	2.5	113	141	4.9	2.	2.9	7	1.05	1.04
LRA	49	51	2.1	3.1	57	124	9.8	3.1	7.7	6.2	1.02	1.49
PV	52	5	2	3.3	102	24	6.1	3.4	3.8	-4.4	1.07	1.13
LRA	48	36	1.9	5	69	230	7.5	5.2	5.6	5.8	1.05	1.57
LRA	54	59	1.9	2.7	93	2.8	5.5	3	3.6	-4.0	0.98	1.48
PV	48	30	1.8	2.7	68	215	7.1	-4.6	5.3	5.4	1.00	0.98
LRA	8	49	1.9	2.8	75	162	8.7	3.5	4.8	-4.5	1.05	1.47
Average PV	49	44	2.1	5	94	193	6.1	3.4	4.0	4.2	1.04	1.05
Average LRA	2	49	2.0	6	74	186	7.4	-4.0	5.4	5.1	1.03	1.50

* Effects of 5-minute infusions of a total dose of 15 milliunits of arginine vasopressin alternately through a peripheral (foreleg) vein (PV) and a catheter in the left renal artery (LRA) in a conscious female dog (16 kg.) during maintained hydration. All data are from the right kidney (The last two columns indicate the ratio of PAH excretion/GFR in the left kidney divided by that for the right kidney.) (From Lauson, 1953.)

The purified arginine vasopressin was kindly supplied by Dr du Vigneaud. The infusions were given every 50 to 60 minutes during the fourth to ninth minute of the period immediately following that corresponding to Column A and just preceding that corresponding to Column B. Sodium para-aminohippurate (PAH, 10 mg.) was included in each vasopressin infusion in addition to the constant infusion of 3.2 mg. of PAH per minute throughout the experiment. Urines were collected every 10 minutes.

† Total solute in urine was calculated as the sum of the millimolar concentrations of urea and creatinine plus twice the sodium and potassium concentrations. Plasma osmolar concentration was taken as 70 milliosmols per liter throughout.

‡ Column A Actual values during second 10-minute preinfusion control periods.

§ Column B Actual values during the first 10-minute postinfusion period. The maximum changes occurred in this period.

‖ Column C Difference between the values of the first postcontrol period and the values in the corresponding preinfusion control period.

Removal of ADH by Kidneys in Dogs

The same principle as was used by Mathe and Altman (1954) and Lauson (1953) has been extended by Lauson (1953) to a study of the removal of ADH by the kidney A polyethylene catheter was inserted retrograde into a secondary or tertiary branch of the *left* renal artery and tied in such a way that the tip opened proximal to the primary bifurcation of the artery This assured that infused vasopressin (du Vigneaud) would uniformly mix with the blood perfusing the non infarcted renal tissue. Antidiuretic effects in the *right* kidney were used for comparing the infusion via a peripheral vein with the identical infusion via the left renal artery Table IV summarizes one of the

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Removal of Oxytocin from the Circulation

The rate of removal of oxytocin was studied in rabbits under urethane anesthesia by Chaudhury and Walker (1957) Single injections of 2 000 000 microunits per kilogram of oxytocin were given and arterial blood was taken at intervals extracted and assayed by the method of Bisset and Walker (1954) on the rat uterus The average half life was 3 3 minutes (see Table VII p 273 for comparative values of half life of vasopressin in man dog and rat) Prior ligation of the renal vessels resulted in prolongation of the half life to 9 7 minutes This suggests that two thirds of the total clearance of oxytocin from the plasma takes place in the kidneys of intact urethane anesthetized rabbits

ESTIMATION OF NORMAL PLASMA CONCENTRATIONS

The following estimations are based on the time course of end organ response during and after intravenous infusions or single injections of vasopressin or oxytocin

Theoretical Considerations

Because few if any valid measurements of the concentration of endogenous ADH or oxytocin in the arterial plasma of man dog or rat under physiological conditions have been made by wholly acceptable bioassay techniques (due to lack of sensitivity and/or specificity) it may be useful and appropriate to attempt to estimate such concentrations indirectly The analysis developed below extends several concepts previously used or implied by such investigators as Dost (1949) in relation to a number of substances and Ginsburg and Heller (1953b) Ginsburg (1954 1957) Hollander *et al* (1957a) Orloff *et al* (1958) and undoubtedly others in relation to ADH

The following grossly simplified assumptions are made (1) As hormone enters the blood it is uniformly mixed in its ultimate volume of distribution (V in milliliters) so rapidly that the process may be considered practically instantaneous for present purposes (2) The virtual volume of arterial plasma completely cleared of hormone per unit time (C in milliliters per minute) is the same for all plasma concentrations of hormone (P in microunits per milliliter) in a given subject This is equivalent to assuming that the quantity of hormone removed per minute is proportional to the concentration of hormone

early experiments Very little if any vasopressin seems to have been extracted by the infused left kidney despite excellent extraction of para aminohippurate (PAH)

It is of course evident from numerous reports that the kidneys excrete antidiuretic material into the urine Since excretion probably represents only a fraction of the hormone removed by the kidney (Ginsburg 1957) some removal should be demonstrable in dogs by Lauson s method of study Again as is shown later (p 271) a relatively low renal as well as hepatic extraction of vasopressin in dogs is consistent with the much lower total clearance in this species as compared to that in rats Experiments to extend this series are under way in the reviewer s laboratory in which a superior method of chronic catheterization of the dog s renal artery is used (Rudolph *et al* 1956) *

Removal of Vasopressin by Muscle

The possibility that skeletal muscle might remove vasopressin in the dog was tested recently (Lauson *et al* 1958) using the same experimental design Submaximal doses of Pitressin had exactly the same antidiuretic effects whether administered via the femoral artery or a foreleg vein This is not in accord with observations of Dicker and Nunn (1957) in rats which led them to conclude that the *pressor* activity of vasopressin diffuses into extracellular fluid and may be adsorbed on muscle from which it could be released in time

In Vitro Studies

That several organs or tissues in addition to the liver and kidneys have the potential capacity to inactivate vasopressin has been demonstrated in several species with *in vitro* methods by a number of investigators (see Heller 1957) However it seems likely (Heller and Zaidi 1957) that the liver and kidneys clear nearly all of the vasopressin from the blood in rats and that these organs also inactivate the hormone promptly and irreversibly

Note added in proof A very satisfactory series of experiments on one dog whose left renal artery was catheterized by the method of Rudolph *et al* (1956) has been completed by Lauson and Bocanegra (1959) The renal extraction of ADH averaged roughly 20 per cent It seems therefore that it will be possible to account for practically all of the ADH clearance in the dog by renal and hepatic removal processes

These equations under the following conditions permit certain statements

During a Constant Rate of Secretion (or Infusion) (1) As time passes the exponential term decreases and approaches zero as a steady state supervenes the concentration of hormone in the plasma

TABLE V Hypothetical Concentrations of Vasopressin in Plasma*

C/V	Per cent of equilibrium concentration			
	50	90	95	99
	min	min	min	min
1 0	0 7	2 3	3 0	4 7
0 10	7	23	30	47
0 05	14	46	60	94
0 025	28	92	120	188

Time (in minutes) to attain the indicated percentages of the equilibrium plasma concentration (P_{eq}) after beginning a constant infusion of vasopressin when C/V is 1 0 0 10 0 05 and 0 025 respectively The times required after cessation of constant infusion of vasopressin for the plasma concentration to decrease to 50 10 5 and 1 per cent of the value attained at equilibrium (P_{eq}) are the same as shown under 50 90 95 and 99 per cent respectively See text for the hypothesis and simplifying assumptions on which this table is based

P therefore approaches a value P_{eq} which is proportional to the rate of secretion (or infusion) and inversely proportional to the clearance and which is equal to their ratio (2) The time required for P to reach the steady state is independent of the rate of secretion (or infusion) (3) The time required to reach plasma concentrations which are given fractions of the equilibrium concentration is similarly independent of the infusion rate and depends only on the rate coefficient C/V i e on the fraction of the volume of distribution which is cleared of hormone per minute (Table V)

After Abrupt Cessation of Secretion (or Infusion) (1) The plasma concentration of hormone is assumed to have attained an equilibrium value P_{eq} by the time the infusion is stopped Considering the moment of cessation as $t_0 = 0$ the equilibrium concentration P_{eq} can now be designated P_0 Equations 2a and 2b then become

$$P = P_0 e^{-C/Vt} \quad (4a)$$

and

$$\ln\left(\frac{P}{P_0}\right) = -\frac{C}{V}t \quad (4b)$$

in the plasma (Unless otherwise specified concentrations of hormone refer to *arterial* plasma all rates and volumes are assumed to be related to surface area or body weight in considering individuals of the same species) (3) The parameters used for evaluating the magnitude of antidiuresis (changes in urine flow urinary osmolar concentration or free water clearance) at each instant reflect in an unvarying way the concentration of hormone in the arterial plasma This obviously requires that GFR $C_{o\ m}$ and all other possible variables remain constant during an observation Delay times and persistence of effect on the tubule cells are assumed to be negligible

Two situations are considered (1) a constant rate of secretion (or intravenous infusion) of hormone (I in microunits per minute) and (2) abrupt cessation of secretion (or infusion) or what is almost the same thing the rapid intravenous injection of a single dose of exogenous hormone

The differential equation

$$\frac{VdP}{dt} = I - CP \tag{1}$$

states that the rate of change in the amount of hormone (microunits per minute) contained in the volume of distribution is equal to the rate at which hormone enters the volume (microunits per minute) minus the amount leaving the volume (microunits per minute) Since V I and C are considered to be constant during an observation the differential equation is readily integrated Integrating and evaluating the constant of integration from the initial conditions $P = P_0$ when $t = t_0$

$$P = \frac{I}{C} - \left(\frac{I - CP_0}{C}\right) e^{-C/V(t-t_0)} \tag{2a}$$

or in natural logarithmic form,

$$\ln\left(\frac{I - CP}{I - CP_0}\right) = -\frac{C}{V}(t - t_0) \tag{2b}$$

If $P_0 = 0$ when $t_0 = 0$ these simplify to

$$P = \frac{I}{C}(1 - e^{-C/Vt}) \tag{3a}$$

and

$$\ln\left(\frac{I - CP}{I}\right) = -\frac{C}{V}t \tag{3b}$$

for example for doses of 1 10 and 100 milliunits the durations were approximately 35 70 and 105 minutes respectively (3) Similar relationships are seen in data on normal dogs in the papers of Samaan (1955) and van Dyke *et al* (1955)

Experimental Application of the Equations At the present time two general applications of these equations are possible (1) those in which physiological quantities of exogenous hormone are given as a single intravenous injection or as a constant infusion and the renal antidiuretic response is observed as a function of time and (2) those in which *very large quantities* of hormone are given and plasma concentrations are directly measured by bioassay in recipient test animals Limited data are available in the literature for both types of applications

From experiments of the first group the expression C/V can be roughly evaluated From experiments of the second category in which constant infusions are used C can be directly estimated as the quotient I/P_q From the measurements of P by bioassay following a single large injection or abrupt cessation of a constant infusion C/I can be estimated provided the curve of disappearance of hormone from the plasma follows a simple semilogarithmic decline Knowing both C and C/I it becomes possible to estimate V and to decide if it is about equal to the plasma volume or if it is significantly greater

Estimation of the Removal Rate Coefficient C/V for Antidiuretic Hormone

We wish to be able to use data indicating the extent of antidiuresis observed during two transient states (during the infusion and after abrupt cessation of the infusion) *to estimate the concurrent* plasma concentrations of the hormone (P) and then to use these derived estimates of P as a function of time to see how well they fit the theoretical expressions derived above If the fit is at all adequate we will have obtained rough estimates of C/I

Estimation of C/V for ADH in Man Data of Hollander et al *(1957a) on normal subjects* Normal men maintained in positive water balance of 1 liter were given constant intravenous infusions of Pitressin at low rates until equilibrium values of submaximal antidiuresis were attained At this time infusions were stopped and urine collections continued The most consistent measure of antidiuresis in their observations was ΔC_{H_2O} (in milliliters per minute per 1 73 M) measured as the difference in C_{H_2O} between the control period of full

which are the familiar equations used by Ginsburg and Heller (1953b) P_0 can also be the initial concentration following a single intravenous injection (2) At the half life time $P = P_0/2$ and $t = (V/C) \ln 2 = 0\,693\, V/C$ (3) The time required for the plasma concentration of hormone to decrease for example to 50 10 5 or 1 per cent of the initial concentration (i e of the equilibrium value at the end of the infusion) is the same as the time required to attain plasma concentrations of 50 90 95 or 99 per cent of the equilibrium value respectively *during* the infusion (Table V) Delay times persistence of effect on the renal tubules and other as yet unpredicted factors may distort this relationship between the two curves The extent of the distortion would be a measure of the quantitative importance of these factors

Validity of the Assumptions and the Resulting Equations In addition to the data to be described subsequently all of which are generally consistent with the assumptions that C is more or less independent of P that V is roughly equal to the plasma volume* and that the decrease of P follows a semilogarithmic course the following evidence may be noted (1) Jones and Schlapp (1936) measured both antidiuretic and oxytocic concentrations in plasma after single intravenous injections of posterior pituitary extracts in spinal cats the curves were approximately semilogarithmic and the half life was 5 to 6 minutes (2) Chalmers and Lewis (1951) reported data on one hydrated normal human subject which are consistent with equation 4b It can be inferred from this equation that the time required for P to fall to a given low value following a single intravenous injection of vasopressin should be proportional to the logarithm of the dose These authors measured the time required for urine flow to return to the level of 10 ml per minute (termed duration of response) following each of a series of vasopressin injections We will assume that P had about the same value at this urine flow in each observation Dosage ranged from 1 to 200 milliunits The relation of logarithm dose to duration of response was linear as predicted by the equation

* See also the section on Nature of the Circulating Hormones (p 241) If a considerable fraction of the circulating hormone were not part of a protein complex this free fraction could diffuse into the interstitial fluid This circumstance would of course greatly complicate the analysis Several consequences would be (1) a longer time to reach the steady state (2) the volume of distribution would have a complicated meaning (3) it would be extremely difficult to predict the changes in P during transient states but (4) since the clearance is known to be relatively large the smaller the free fraction the more closely the curve of disappearance of ADH from plasma would approximate a semilogarithmic function Moreover if the clearance were not independent of plasma concentration the analysis would be further complicated

by substituting values of ΔC_{H_2O} I and mid period t from a total of 21 postinfusion periods in four subjects *

With the same assumptions data obtained from the beginning of a vasopressin infusion to the attainment of equilibrium could be used to estimate C/V Unfortunately adequate data of this type are not available at present experiments are under way in the reviewer s laboratory to obtain such data in normal dogs

TABLE VI Estimates of the Pitressin Removal Rate Coefficient C/V

Subject	Rate of preceding infusion I (μU/min /1 73 M)	Number of periods calculated	C/V	
			Average	Range
T C	147	9	0 043	0 026 0 053
J A	62	5	0 048	0 031 - 0 068
D D	21	3	0 068	0 029 0 111
R S	15	4	0 031	0 023 0 042
		Weighted average	0 046	

* These estimates were calculated from selected normal human data of Hollander et al (1957a) Equilibrium data from these four subjects as well as from Subjects D R and W M were used to compute the constants in equation 5a

Data of Lauson (1951) on one normal human subject Figure 3 (p 252) shows the changes observed in four experiments in the endogenous creatinine urine/plasma concentration ratio U/P following abrupt cessation of Pitressin infusions one at a just maximal rate and three at supramaximal rates It is assumed that equilibrium was attained in each experiment therefore $P_{eq} = I/C$ It is further assumed tentatively that C was constant during each experiment and that both it and V were about the same on each of the four occasions For the rates of 200 700 3167 and 4083 microunits per minute the approximate times required for U/P to decrease to an arbitrarily chosen low value of 50 were 47 75 95 and 107 minutes respectively Assuming now that the plasma concentration (P') corresponding to U/P of 30 was the same in each observation we can make a reasonable guess that this value of P was roughly equivalent to that which would have

Hollander and associates kindly made these unpublished data available to the reviewer The reviewer is indebted to Dr Josiah Macy Jr of the Department of Physiology Albert Einstein College of Medicine for suggesting the mathematical approach used in the analysis of these data of Hollander *et al* (1957a)

diuresis and the equilibrium period of an infusion The relationship between the equilibrium values of ΔC_{H_2O} and the corresponding rates of the infusion (I in microunits per minute per 1 73 M) was found by these investigators to be reasonably well described by an equation of the form $1/\Delta C_{H_2O} = a + b\,(1/I)$ Following this lead the reviewer selected nine steady state observations in six of their subjects in which C_{osm} ranged only from 1 3 to 2 7 ml per minute per 1 73 M² in which changes in C_{osm} between control and infusion periods did not exceed 0 4 ml per minute per 1 73 M² and in which I ranged from 15 to 147 microunits per minute per 1 73 M² The following equation was fitted empirically by the method of least squares

$$1/\Delta C_{H_2O} = 0\,0707 + 3\,77(1/I) \qquad (5)$$

which on rearrangement is

$$\Delta C_{H_2O} = \frac{I}{0\,0707I + 3\,77} \qquad (5a)$$

But at equilibrium $P_{eq} = I/C$ where C is expressed in milliliters per minute per 1 73 M² Hence $I = CP_{eq}$ and

$$\Delta C_{H_2O} = \frac{CP_{eq}}{0\,0707CP_{eq} + 3\,77} \qquad (6)$$

After the infusion is stopped we have postulated that

$$P = P_0 e^{-C/Vt} \qquad (4a)$$

where $P_0 = P_{eq}$ We next assume that the *functional* form of equation 6 derived from *steady state* relationships will apply with reasonable approximation to the transient state following cessation of the infusion Therefore the value of P from equation 4a is substituted for P_{eq} in equation 6

$$\Delta C_{H_2O} = \frac{CP_0 e^{-C/Vt}}{0\,0707C(P_0 e^{-C/Vt}) + 3\,77} \qquad (7)$$

Since P_0 is the same as the equilibrium value at the end of the infusion and is equal to I/C equation 7 becomes

$$\Delta C_{H_2O} = \frac{Ie^{-C/Vt}}{0\,0707Ie^{-C/Vt} + 3\,77} \qquad (8)$$

Table VI summarizes the range of estimated values for C/V obtained

decrease in plasma concentration 14 minutes was needed According to equation 4a C/V is approximately 0 099 in this case

From the reviewer s own observations and from data published by a number of investigators showing the time course of urine flow or other antidiuretic parameters following a single submaximal injection or cessation of a constant submaximal infusion of posterior pituitary extracts it is evident that almost complete return of diuresis usually requires 40 to 50 minutes in the dog Assuming that the clearance of vasopressin is 98 to 99 per cent complete in this time C/V can be estimated to be roughly 0 10

Using an unphysiologically high rate of infusion of Pitressin Lauson *et al* (1958) directly estimated *clearance* in a dog anesthetized with pentobarbital Pitressin was infused at a rate of 33 333 microunits per minute (2500 microunits per minute per kilogram) for 3 hours to assure complete equilibration Arterial blood was drawn near the end of this period and mixed with heparin a portion was centrifuged and both samples were stored in ice water Both whole blood and plasma were assayed for ADH content under unusually favorable circumstances in a trained hydrated dog during an interval of 1 to *3½ hours after the blood was taken from the donor There was no* apparent loss of ADH activity in this interval The assays indicated a plasma concentration of 420 microunits per milliliter none of the Pitressin seems to have been adsorbed by or penetrated into the red blood cells Dividing the infusion rate by the plasma concentration (33 333/420) gives a clearance value of 79 ml per minute (6 1 ml per minute per kilogram) If the infused Pitressin had been distributed ex clusively in the plasma volume (measured by T 1824 dilution as 740 ml) a value of 0 11 for C/V would be indicated For comparison the renal plasma flow in this donor dog measured as the clearance of PAH was 190 ml per minute Thus even if all of the Pitressin clearance takes place in the kidneys of dogs an extraction ratio of no more than about 40 per cent would be indicated If about half of the removal occurs in other organs such as the liver even smaller extractions are probable

Therefore in the dog possibly less than 20 per cent of the vasopres sin content is removed from the blood perfusing the kidneys and liver These data seem to be consistent with those cited in the preceding paragraphs and give some support to the assumptions that the apparent volume of distribution may be no greater than the plasma volume

obtained at equilibrium during an infusion one eighth as great as the rate just sufficient to induce maximum antidiuresis i e 25 micro units per minute Thus this estimated P' is $25/C$ Substituting this value as well as each of the four infusion rates and the respective times into equation 4b yields four solutions for the value of C/V ranging from 0 044 to 0 051 (average 0 047) Taking values for P' which are twice and one half as great as $25/C$ and similarly substituting we obtain values for C/V of 0 029 to 0 044 (average 0 037) and 0 054 to 0 059 (average 0 056) respectively The real value certainly lies some where in this total range

The estimates of C/V for normal man based on the data of Hol lander *et al* (1957a) and Lauson (1951) are therefore of the order of 0 03 to 0 06 that is about 3 to 6 per cent of the volume of distribution is cleared of Pitressin per minute If V were taken to be equal to one two or three times the plasma volume the corresponding estimates of C would lie somewhere between 90 and 540 ml per minute The crucial importance of knowing the volume of distribution is revealed here because without this knowledge such estimates of clearance must be limited to wide possible ranges

Even this broad estimate of clearance fairly definitely excludes the possibility that the liver and kidneys in man remove large fractions of vasopressin from the blood perfusing them If both of these organs completely extracted the vasopressin from the plasma passing through them the maximum possible clearance would be the sum of hepatic and renal plasma flows (approximately 1500 ml per minute in normal man) Then even if V included the whole extracellular fluid (about 11 000 ml) C/V would need to be as great as 0 14 so high a value is improbable It is likely therefore that the clearance of Pitressin in man is much smaller than the sum of liver and kidney plasma flows

Estimation of C/V for ADH in Dogs Shannon s 14 1 kg normal dog E (1942b) was hydrated initially with 50 ml per kilogram and given a constant infusion of Pituitrin at 83 3 microunits per minute At near equilibrium the urine osmolar concentration was 1000 milli osmols per liter Then an infusion four times greater (333 microunits per minute) was given and U_m reached 1710 milliosmols per liter at what must have been nearly equilibrium By interpolation the time required for U_m to decrease to 1000 milliosmols per liter (equivalent to the value attained during first infusion) after the second infusion was stopped was roughly 14 minutes Thus for an estimated fourfold

a concentration of approximately 10 microunits per milliliter If equation 4a is valid, a value for C/V of the order of 0 5 can be calculated for these two rats In four other rats given 200 to 1000 microunits per minute per kilogram for 30 to 60 minutes plasma taken 3 minutes after cessation of the infusion contained less than 3 5 microunits per milliliter this is consistent with a value of C/V of about 1 0 All things considered these data of Dicker s can be said to confirm the observations of Ginsburg and Heller

TABLE VII Estimates of Vasopressin Clearance (C) Removal Rate Coefficient (C/V) and Half life in Normal Man Dog and Rat

Species (and number of subjects)	Average body weight	Vasopressin clearance	Apparent volume plasma	C/V	Half life	Author
	kg	ml/min	ml		min	
Man (4)	80 0	110 24	3600	0 031 0 068†	10 22	Hollander et al (19[illegible])
Man (1)	4 0	170 180	3300	0 037 0 0 8†	12 19	Lauson (1951)
Dog (1)	14 1	77	7 5	0 099†	6 9	Shannon (1942)
Dog (many)	—	—	—	ca 0 10†	6 9	[illegible]
Dog (1)	13 0	9†	740	0 11	6 3	Lauson et al (1958)
Rat ( )	0 5	6 1	7 6‡	0 81†‡	0 85‡	Ginsburg and Heller (195 b)
Rat (4)	0 25	6 2	7 6‡	0 8 †‡	0 8 ‡	Ginsburg (1954)
Rat (6)	0 90	9 8†‡	9 8‡	1 0‡	0 70‡	Ginsburg (19 7)
Rat ( )	0 200	ca 3 4	6 7	ca 0 5†	ca 1 4	Dicker (1954)
Rat (4)	—	—	—	ca 1 0 or over	ca 0 7	Dicker (19 4)

As[illegible]med to b 45 ml kg [illegible] man 55 ml /Kg for dog and 3 ml /kg [illegible] Th[illegible] lue in Lauson s dog was meas[illegible] ed with E[illegible] B[illegible] The [illegible] me of vasop[illegible] es in dist ibutio (V) [illegible] as[illegible] umed to be equal to th plasma [illegible] See text f[illegible] detail

† Indi[illegible] tes th primary meas[illegible] nt [illegible] est[illegible] ate F[illegible] exampl[illegible] [illegible] C/V is the prim ry e timate C [illegible] de[illegible] ed from th[illegible] and th assumed plasm volu e. If C i the prim ry estimat C/V is [illegible] milarly deri ed

‡ A[illegible] ow[illegible] tim te All oth[illegible] est m tes [illegible] e th[illegible] [illegible] f the eviewer

Prediction of the Physiological Ranges of Antidiuretic Hormone Concentration in Plasma

The essential conclusions concerning the estimated magnitude of the clearance of the antidiuretic activity of exogenous vasopressin and of the removal rate coefficient C/V for man dog and rat are summarized in Table VII The tentative and approximate nature of these estimates must be re-emphasized The comparative values of C/V show a remarkable range In round numbers man clears in each minute about 5 per cent of the volume in which Pitressin is distributed the dog clears about 10 per cent while the rat clears nearly 100 per cent

and that the Pitressin clearance is approximately constant for all plasma concentrations of ADH

Estimation of C/V for ADH in Rats Ginsburg and Heller (1953b) gave large intravenous injections of Pitressin (100 milliunits per hundred grams or 1 000 000 microunits per kilogram) during a 20 second period to rats anesthetized with ether and at several intervals took a small volume of blood for bioassay of the concentration of Pitressin As mentioned previously these data indicated a semilogarithmic decrease of blood concentration in which C/V averaged 0 81 On the assumption that V was the same as the plasma volume (estimated as 3 37 ml per hundred grams) they calculated a clearance of 2 7 ml per minute per hundred grams (27 ml per minute per kilogram) In a parallel study a year later Ginsburg (1954) found a value of 0 82 for C/V in a group of sham operated but otherwise intact rats In both experiments the curve of the logarithm of plasma concentration against time extrapolated to zero time indicated a value for P_0 equal to the dose injected divided by the plasma volume

Ginsburg (1957) also measured the clearance of Pitressin by the method of constant infusion and bioassay of the plasma concentration before the end of the infusion In six *unanesthetized* rats given 30 000 to 60 000 microunits per minute per kilogram for 90 minutes the total clearance of Pitressin ranged from 1 8 to 4 8 ml per minute per hundred grams (average 34 ml per minute per kilogram) If V were equal to the plasma volume C/V would be 1 0 a value which closely agrees with the earlier estimates of C/V of 0 81 and 0 82 These data of Ginsburg and Heller support the assumption that V is approximately equal to the plasma volume and that C is constant over a wide range of P

On the average the *total* Pitressin clearance was 6 8 times as great as the concurrent inulin clearance (Ginsburg 1957) If it is assumed that the renal plasma flow (not measured) was three or four times as great as the inulin clearance under these conditions a nearly *complete* extraction of Pitressin by the kidneys would have to be postulated to account for half of the total clearance leaving the other half to occur elsewhere presumably in the liver These data are consistent with those of Ginsburg and Heller (1953b) which were described previously

Dicker (1954) made brief mention of an observation in two rats which is pertinent Pitressin was infused intravenously into these 200 gm rats for 60 minutes at the nearly physiological rate of 500 microunits per minute per kilogram Blood was removed 2 minutes *after* cessation of the infusion and bioassay of the plasma indicated

in the rat as in man whereas C/V is about twenty times as great in the rat This suggests — most tentatively — that despite the great differences in C/V the physiological range of plasma concentrations may be only slightly higher in the rat than in man (Table IX)

Estimation of Plasma Concentrations and C/V for Oxytocin in Women during Parturition

The data of Caldeyro Barcia *et al* (1957) referred to earlier (p 255) indicate that in normal women in late pregnancy the rates of infusion of oxytocin required to increase uterine activity to the average level seen at the beginning and end of the first stage and during the second stage of labor are 1 to 2 8 and 16 milliunits per minute respectively If the volume of distribution is assumed to equal the plasma volume and if C/V is about 0 06 (half life 10 to 12 minutes) as estimated by the reviewer from their data on 1 patient (Record No 576b) these infusion rates would be expected to increase the plasma concentration of oxytocin over endogenous levels by roughly 5 to 10 40 and 80 microunits per milliliter respectively These estimates of oxytocin concentration occurring during labor may be compared to the probable concentrations of ADH (1 to 5 microunits per milliliter) required for submaximal to maximal antidiuresis in human subjects (Table IX)

BIOASSAY OF ENDOGENOUS VASOPRESSIN AND OXYTOCIN IN BLOOD PLASMA OR SERUM

Bioassay Methods

Because all measurements of plasma concentrations of the hormones depend on bioassay procedures it is essential that these methods be as specific sensitive and precise as possible The reviewer strongly endorses the opinion of van Dyke and associates (1955) that whenever a biological fluid is to be assayed in a living animal the *intravenous* route of administration should be used Details of the best methods for most of the biological activities have been ably described by these authors and by Thorn (1958a)

Results of Bioassays of Blood Plasma or Serum for Antidiuretic Activity

Many of the available data on antidiuretic assays of blood plasma or serum from man dog rat and kangaroo rat are summarized in Table X The condition of the subject from whom the blood was obtained is

If the many assumptions and resulting estimates are even roughly correct several important consequences ensue *First* the extraordinarily large clearance in the rat apparently requires that both liver and kidneys extract nearly all the vasopressin from the blood perfusing

TABLE VIII Estimate of Approximate Rates of Infusion of Vasopressin Required to Achieve an Arterial Plasma Concentration of 1 µU/ml at Equilibrium

Species	Body weight	Surface area	Estimated pressin clearance (C)	Approximate plasma volume (V)	C/V	Required infusion rate		
	Kg	M^2	mL/min.	ml		µU/min.	µU/min./kg.	µU/min./M^2
Man	70 0	1 73	150 0	3000 0	0 05	150 0	2 2	87
Dog	20 0	0 75	100 0	1000 0	0 10	100 0	5 0	133
Rat	0 25	0 036	8 4	8 4	1 0	8 4	33 6	233

B ed th as mpti n that V is eq l to th plasm l m and th t C/V has th al di ted.

TABLE IX Predicted Physiological Ranges of Antidiuretic Hormone Concentration in Plasma*

Species	Body weight	Surface area	Approximate range of infusion rate			Expected range of arterial plasma concentration†
	kg	M^2	µU/min	µU/min /Kg	µU/min /M^2	µU/ml
Man	70 0	1 73	150 750	2 11	90 400	1 5
Dog	20 0	0 75	00 1000	10 50	270 1330	2 10
Rat	0 25	0 036	25 50	100 200	700 1400	3 6

*Inf i ns t f a p i ar th estim t d t b equi ed to h ly j t max mal (++ to ++++) antidi l t q lib i m (Tabl III) i hyd t d man d g and t f C/V 0 05 0 10 and 1 0 r pecti ly d if V i eq l to th pl m l m N mal GFR and di ry t f m l l an as med

†Bas d m lecul w ight f 1084 f rgi i op i nd pot ncy f 400 nit p millig m (Tabl I) plasm co t tio f 1 m t p mill l t q i lent to abo t 1 0 10 $^{-9}$ mi romol p millil t

them whereas in man and dog these extraction ratios must be very much smaller *Second* to attain a given plasma concentration (for example 1 microunit per milliliter) during a steady state considerably more hormone must be infused or secreted per minute relative to body weight or surface area in the rat than in man (Table VIII) *Third* although the estimates of the rates of infusion required for just maximal antidiuresis in the three species summarized in Table III (p 250) cannot be compared too closely because of wide differences in conditions of the experiments (water loading C_{osm} GFR etc) it seems that infusion rates per square meter required for this degree of kidney response are probably of the order of only five times as great

7	Normal wom (10)†	Ant c bital v i ac d alc h l e tra t of blood	*Hydr ted th olized ts*	N t stat d	6 to 28 (7) *9 to 22 (3) B)*	Robinso t al (1957)
b	Pregna t w me (5)†	A t c bital vel a id lc hol xt act of blood	Hydrated ethan li d r ts	Not tated	38 (B)	Robin n t al. (1957)
c	W me du i g parturitio (2)†	A t c bital a d lc h l tr t of blood	Hydrat d ethan lized t	Not stat d	42 (B)	Robinson t L (1957)
d.	La tati g w n *b fore s kli g (5)†*	Ante bital in a d alc h l t t f bl od	*Hydr t d thanoliz d* r ts	Not tat d	35 (B)	Robinson t l (1957)
	S m d i g s kling *(5)†*	Ant bital i a d alc hol tr ct f blood	Hydr ted tha li d ts	Not tated	66 (B)	*Rob nson* t al (1957)
8a.	Child with phr tic ed m 15 h *d hydr ti* (1)	Ante bital in, indw lli g dl	Child w th diab te i ipidus (hydr ted)	75	6 (B)	Ba tt t al (1952)
b	S m child, afte ACTH l duced d re is 15 h d hydration	Art bital i indw lli g dle	S m child w h diab t s insipidu (hydrated)	90	*(B)*	B *nett t al* (1952)
9	Infants with kwashi kor and p tle t, with *cirrhosi a d a ley†*	Inte nal j gular i (o local a e th la)	N t tated, pr bably hydr ted una e theti d ts	*Not stated*	11 to 152 *(P)*	*H ller (1956)* d H ll r a d Schn d (1957)
B.	Dog					
1	N mal (24)†	Not *tated p bably* xte al jugular vei	Dogs with di b tes insipid d hydr t d 3 h r	Up to 50	2 to 10 (23) p iti b t l not stated (1) (B)	Hare et al (1945)
2	N mal 48 hr d hydrati	E ternal jugular i	Dog with diabet s i sipid d hydrat d 3 ho r	10	10 (B)	*Hare et al (1941)*
3a	N m l, 24 hr d hyd ati (8)	E te nal jugul r vein	Hyd at d ethanolized ats	0 65	80 (a g 45 to 105) (P)	Blackmo e and Che ter (1956)
b	Diabet s insipidu (4)	Exte al j gular n	Hyd ted ethanoli d r ts	*0 65*	*20 (P)*	*Bl ckm or* a d Che ter (1956)
4	N mal (19 5 kg) thir ti g (1)	110 ml withdra f om nspe *tiled in,* d stored at nsp cifi d te perat re for 1 hr	*Reinje t n i to me dog afte* hydr t o	110	ca 2 (B)‡	Marx (1930)

TABLE X. Concentrations of Antidiuretic Substance in Blood Plasma or Serum as Assayed by Intravenous Injection in Hydrated Recipients

Donor and state (number of subjects)	Site of blood and method	Assay method and animal	Volume injected ml	Average concentration µU/ml	Author
A Human subjects					
1 Normal 15 hr dehydrated (1)	Internal jugular vein, via catheter in antecubital vein, heparinized, stored in ice water	Reinjection during 5 minutes into hydrated donor 4 hr later	100	6 (B)	Lauson et al (1951)
2 Normal 12 hr dehydrated (6)	Antecubital vein serum at room temperature	Reinjection into hydrated donor 1 hr later	*3 to 17*	*100 (S)*	Lewis (1953)
3 Patients thiopentone anaesthesia (9)†	Internal jugular vein acid alcohol extract of blood	Hydrated ethanolized rats	Not stated	10 to 30 (7) 12 (1) 20 (1) (B)	Bisset and Lee (1957)
4 Normal male (3)†	Internal jugular vein local anaesthesia	Not stated	Not stated	11 (1) 20 (2) (P)	Heller and Schniede (1954)
5a Normal hydrated (5)	Antecubital vein (paraffined needle in oil)	Hydrated normal human recipient	175 to 400	4 (4) 5 to 10 (1) (B)‡	Brun et al (1946)
b Man, 5 minutes post syncope (3)	Antecubital vein	Hydrated normal human recipients	200 to 400	*5 to 10 (B)‡*	*Brun et al (1946)*
6a Normal man in winter season, dehydrated 3 hr (4)	Antecubital vein	Hydrated ethanolized rats	0 2/100 gm	20 (P)	MacFarlane and Robinson (1957)
b Same after 4 hr in hot room	Antecubital vein	Hydrated ethanolized rats	0 2/100 gm	20 (3) 30 (1) (P)	MacFarlane and Robinson (1957)
c Same man in summer season dehydrated 3 hr	Antecubital vein	Hydrated ethanolized rats	0 2/100 gm	37 (P)	MacFarlane and Robinson (1957)
d Same after 4 hr in hot room	Antecubital vein	Hydrated ethanolized rats	0 2/100 gm	112 (P)	MacFarlane and Robinson (1957)
Same after walking 4 miles/hr in hot room [illegible] 10 min	Antecubital vein	Hydrated ethanolized rats	0 2/100 g[illegible]	100 to 200 (P)	MacFarlane and Robinson (1957)

5 a. N rm l, th r a the a 9)†	H art p t r d capillatio (tr nk blood)	Hydrated eth l z d ats	Up to 1 0	290 42 (S)	An es a d Dyk (1952)
b 3 t 4 weeks p t hyp phy cto y ther a th i (12)	Tr k blood aft pid d c pitat	Hydr t d eth n li d ts	Up t 1 0	20 (S)	A and va Dyk (1952)
6a. No al, th r an th ia (4)†	1 t 2 ml arte ial bl od	Hydrated a esth li d ts	Up t 1 0	50 (B)	Gi burg nd Heller (1953)
b Sa e co dition (4)	1 ml exte nal j gul blood	Hydrated thetiz d rats	Up to 1 0	200 (B)	Ginsb g and Hell r (1953)
Same co d tio (4)	5th ml f c e l e 1 ml. withdr w ls f arte ial blood	Hydrated na e th li d r t	Up to 1 0	450 (B)	Gl sb rg and Hell r (1953)
d. San e c ditio (4)	5th ml. f c es 1 L withdrawal of ext nal j gul r blo d	Hydrated unan thetl ed ats	Up to 1 0	3500 (B)	Gl b b d H ller (1953a)
e Sa n dit n (6)	4 t 5 ml rterial bl d (all i n withdr w l)	Hyd ated nane th tiz d ats	Not t t d	270 (P)	Gl lsb b d H ller (1953a)
f S m c nditi (4)	Sam a 6e	Hydrated na esth tiz d rats	Not tat d	280 (S)	Gin b b d H ll (1953a)
g Same c ditio (8)	S me a 6e b t f m ter al jug l ei	Hydrat d a e thetized ts	N t tat d	1300 (P)	Ginsb g a d H ller (1953a)
h. Sam co dition (4)	Sam a 6e b t a ly fr e l a d h p t ein	Hydr ted nane th tiz d ts	N t t t d	100 (P)	Gin b g a d Hel er (1953a)
i S m o diti (5)	S a 6 b t f om inf i r v a c a w th e l a d hep tic o tfl w cl d d	Hydrated a esthetized ats	N t tated	310 (P)	Gl b g d Hell r (1953)
7a. Adr n l ctomiz d 6 day eth an sth i (4)†	1 1 arterial blood	Hydrated nare th tiz d ats	Up to 1 0	100 (B)	G nsbu g (1954)
b Sa e ats (4)†	3 d n l f s c lve 1 ml withd wals of a terial blood	Hydr t d nane th ti ed ats	Up t 1 0	1240 (B)	Gi b g (1954)
8 No mal, th r an sthesia, i tra rdi c I j cti of 0 5 mL of 0 45 M N Cl	1 ml blood, probably by h art p ctu e	Hydr ted Uhanoliz d ats	0 2 to 0 5	1000 t 3000 (S)	Th r nd Silver (1957)

TABLE X Concentrations of Antidiuretic Substance in Blood Plasma or Serum as Assayed by Intravenous Injection in Hydrated Recipients (*Continued*)

Donor condition (number of subjects)	Site of blood withdrawal	Assay method and animal	Volume injected ml	Average concentration µU/ml	Author
5 Normal Intravenous infusion of 0 45 M NaCl at 10 ml/min for 45 min	Not stated, probably external jugular vein blood withdrawn at specified time after infusion	Dog with diabetes insipidus dehydrated 3 hours	40 to 50	2 to 10 (B)‡	Chambers et al (1945)
6a Chloralose anesthesia intracarotid infusion of 0 5 M NaCl at 4 to 8 ml/min. (5)	External jugular vein blood withdrawn during infusion	Hydrated unanesthetized dogs	10	100 or more (B)	Ames, Moore and van Dyke (1950)
b Same conditions (2)	Internal jugular vein blood withdrawn during infusion	Hydrated unanesthetized dog	2	500 (B)	Ames Moore and van Dyke (1950)
c Same conditions (1)	Femoral vein blood withdrawn during infusion	Hydrated unanesthetized dog	Not stated probably 10	Probably 25 (B)	Ames Moore and van Dyke (1950)
d Chloralose anesthesia (5)	External jugular vein blood withdrawn before infusion	Hydrated unanesthetized dogs	10	25 (B)	Ames Moore and van Dyke (1950)
C Rats					
1 Normal unanesthetized, tranquil (4)†	[illegible] blood from external jugular vein via indwelling catheter	Hydrated anesthetized rats	1/100 g	100 (4) (B)	Ginsburg and Heller (1953a)
2 Same condition (14)†	Trunk blood after rapid decapitation	Hydrated ethanolized rats	0 5 to 1	20 to 40 (S)	Ames and van Dyke (1952)
3a Same condition (8)†	0 3 to 0 4 ml/100 gm of external jugular blood via needle	Not stated, probably hydrated rat	Not stated	36 to 55 (7) ca 50 (1) (B)	Schlieden and Blackmore (1956)
b Same but excited (5)	0 3 to 0 4 ml/100 gm of external jugular blood via needle	Not stated, probably hydrated rats	Not stated	80 to 250 (B)	Schlieden and Blackmore (1956)
4a. C [illegible] 22 C (6)†	Trunk blood after rapid decapitation	Hydrated ethanolized rats	0 4	27 ± 18 (P)	Robinson and M F [illegible] (1956)
b 2 [illegible] 40 C (9)	Trunk blood after rapid decapitation	Hydrated ethanolized rats	0 4	30 16 (P)	Robinson and M F [illegible] (1956)
4 h [illegible] 40 C (5) [illegible]	[illegible]	[illegible]			

briefly described and ranges from physiological (for example 12 hours of dehydration) through pharmacological (for example ether anesthesia) to frankly pathological (for example the nephrotic syndrome) In some cases the authors did not describe the state of hydration in their normal subjects The site of blood removal is indicated because many assays were carried out on venous blood draining the neurohypophysis (internal or external jugular vein) and therefore represent concentrations which are an unknown number of times as great as *those in arterial blood Greater validity can be ascribed to observations* in which blood or plasma is assayed by prompt injection intravenously without modification into a member of the same species than to those in which blood or plasma is subjected to an extraction procedure or is injected into a member of another species Only data from intravenous assays are presented Because urine flow was the sole parameter measured in most of these bioassays it remains uncertain whether nonspecific effects on GFR or C_{m} might have occurred in some instances which would modify or even vitiate any conclusions

The purpose of this tabulation is not so much to present conclusive data on the plasma concentrations of ADH in the various normal and abnormal states Rather *it is merely to suggest the probable order of* magnitude and the direction of possible changes and to point out how little is known with certainty

Physiological Dehydration Simple dehydration under ordinary environmental temperature and sedentary activity would seem to be the condition most readily standardized for the purpose of measuring an upper physiological limit of ADH concentration with which the concentrations observed in unphysiological or disease states could be properly compared Preferably data on the subject s concurrent urine flow U_{m} C_{m} and C_{H_2O} should also be obtained at the time of the blood sampling

In man the majority of such physiological concentrations were less than could be detected even when internal jugular blood or plasma was used The lowest limit of detectability (6 microunits per milliliter) was in the study of Lauson *et al* (1951) 100 ml of internal jugular blood was withdrawn after 15 hours of overnight dehydration in the winter season heparinized stored in ice water and reinjected 4 hours later when the subject was in maintained maximal water diuresis The concentration was less than 6 microunits per milliliter of blood (less than 10 to 12 microunits per milliliter of plasma) If it is assumed that the internal jugular blood flow is diluted sixfold in mixing with

TABLE X Concentrations of Antidiuretic Substance in Blood Plasma or Serum as Assayed by Intravenous Injection in Hydrated Recipients (*Continued*)

Dono co diti (n mb f bj t)	S te f bl d ithd w l	As y m thod d animal	V l m injected ml	A ag c ent tio μU/ml	A th
D Kangaroo rats (Dipodomys merriami)					
1a *Normal*, unanesth ti ed, tranquil b t me app ehension (20)	*Trunk blood afte* apid d capitation	Hydrated ethanol ed ts	Up to 1 0	40 (8) 40 to 190 (12) (S)	Ames and van Dyke (1952)
b Normal, anesthetized (4)	Heart p ncture	Hydr ted ethan l ed rats	Up t 1 0	750 to 2250 (S)	Ames and van Dyke (1952)
c Ether anesthesia (16)	H a t p ncture *r decap tation*	Hydrated ethanoliz d rats	Up t 1 0	380 to 2000 (S)	Ames a d van Dyke (1952)

Th ymbol indi t that th co t t w b l w th tat d lim t f d t tabil ty blood (B) pl m (P) Serum (S)

† Stat f hyd ti w t des b d

‡ He lew estimate th did not alib t th espons te m f as p es ta d d

methods were less than adequate it is significant that in the majority of cases no antidiuretic activity was detected in the recipient when 500 ml of citrated blood was infused during a 40 minute period

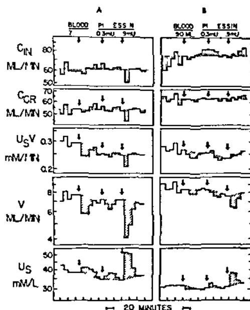


FIGURE 5 Assay of ADH in antecubital venous blood of a 3½ year old child with nephrotic syndrome during a period of severe and increasing edema (A) and on the ninth day after the last dose of a course of ACTH which had resulted in diuresis (B) Blood was removed without emotional disturbance or pain after a 15 hour period of fluid deprivation The assays consisted of injecting the donor blood intravenously into a child with severe untreated diabetes insipidus and comparing the response to that following intravenous injection of Pitressin Arrows indicate injections of blood or Pitressin into the recipient The data shown describe changes in the recipient child Measurements included inulin clearance (C_{I}) specific endogenous creatinine clearance (C_{CR}) total urinary solute excretion approximated by the sum of excretions of urea sodium potassium chloride phosphate creatinine and inulin (U_SV in millimols/min) urine flow (V) and total urinary concentration of the sum of the same solutes (U_S) Comparison of the shaded areas in lower row indicates that the blood contained the equivalent of about 6 μU/ml of antidiuretic hormone during the edema phase (A) and none (<2 μU/ml) during diuresis (B) Measurements in the *donor* child on these two occasions respectively were body weight 21 0 (increasing) and 18 8 (decreasing) kg C 15 6 and 37 4 ml/min (35 and 83 per cent of normal) U_SV 0 069 and 0 121 mM/min V 0 149 and 0.246 ml/min U_S 460 and 489 mM/L (From Barnett *et al* 1952)

Examination of the data in Table X reveals that pain excitement ether anesthesia strong osmotic stimulation and especially hemorrhage are capable of causing the release of large amounts of ADH In some

the resting cardiac output an arterial plasma concentration of less than about 2 microunits per milliliter would be indicated This is consistent with the estimates given in Table IX

In the *dog* the observations of Hare and associates (1941 1945) reveal concentrations of less than 2 to less than 10 microunits per milliliter in external jugular blood after 48 hours of dehydration Marx s (1930) observation indicates a concentration in the lower part of the estimated range given in Table IX The values reported by Blackmore and Chester (1956) for external jugular plasma therefore seem to be too high but no adequate explanation can be suggested at this time

In the normal *rat* the concentrations in external jugular blood or serum and in plasma or serum from trunk vessels were too low to be detected in the studies of Ginsburg and Heller (1953a) Ames and van Dyke (1952) and Schnieden and Blackmore (1956) The concentration in the control rats of Robinson and MacFarlane (1956) however averaged 27 microunits per milliliter which is somewhat higher than expected from the estimates in Table IX No mention of dehydration was made in any of these reports Assays following standardized periods of dehydration are clearly needed in the rat

The data on exposure to heat in man (MacFarlane and Robinson 1957) and the rat (Robinson and MacFarlane 1956) involve a factor of dehydration and increase in plasma osmolar concentration and are qualitatively consistent with the idea of increased ADH secretion in this circumstance The human data were obtained by injecting human plasma into rats and are therefore probably less surely specific than the data on assay of rat plasma in rats

Other Conditions Most of the observations shown in Table X are self explanatory and require no comment However the data on pregnant parturient and lactating women of Robinson *et al* (1957) should be noted as possibly suggesting some nonspecificity in the extraction or assay The most adequate bioassay of ADH in blood in a human disease appears to be that of Lauson *et al* (1952) in which blood from a child with the nephrotic syndrome was injected into a recipient child with severe diabetes insipidus The data which were published in graphic form in the review by Barnett *et al* (1952) are reproduced in Figure 5

Historically the first serious attempt to measure ADH in human blood by direct injection of whole blood into hydrated normal human recipients was that of Page (1938) Although by present standards his

lation and injected it intravenously into a second lactating goat A definite ejection of milk resulted in the recipient

These few observations on the oxytocin content of blood are of considerable importance qualitatively in helping to establish the mechanisms of action of the hormone but they do not provide ade quate quantitative estimates of physiological concentrations Ap parently no estimates of oxytocin in human plasma have yet been made by wholly acceptable methods

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instances the resulting plasma concentrations are astonishingly high Ginsburg and Brown (1957) discussed the possible importance of such ADH liberation in supporting the circulatory system during severe hemorrhage

It is evident that more sensitive assay methods are required before more progress can be made in estimating arterial plasma concentrations of ADH in man and other species under various physiological and pathological conditions A promising beginning has been made in the reviewer s laboratory on a study of endogenous ADH concentrations in the plasma of dogs Injection of the plasma directly into one renal artery of the assay dog via a chronically implanted catheter (Rudolph *et al* 1956) permits detection of concentrations 5 to 10 times less than those detectable by the usual intravenous route of administration

Results of Bioassay of Blood for Oxytocic and Milk ejecting Activities

Oxytocic activity of cow s blood has been tested by adding the blood to the Tyrode s solution perfusing an excised cow s uterus Hays and van Demark (1953) removed 250 ml of external jugular blood before and after the mating stimulus had been applied artificially in three cows None of the prestimulation samples contained demonstrable oxytocic activity whereas the three poststimulation samples induced a series of contractions the pattern of which resembled that caused by perfusion with oxytocin However the concentration in the blood was not reported in terms of oxytocin units

Fitzpatrick and Hughes (1956) performed a similar experiment in cows but they extracted the external jugular blood and assayed the extracts on the superfused rat uterus Before the mating stimulus the blood contained 120 to 300 microunits per milliliter whereas after the stimulation the concentration increased to 420 to 850 microunits per milliliter The activity was destroyed by thioglycollate and neither atropine nor lysergic acid diethylamide blocked the action

Petersen and Ludwick (1942) perfused isolated udders of cows with external jugular blood withdrawn from lactating cows before and after application of the milking stimulus Perfusion of 1 liter of post stimulation blood caused milk-ejection in the isolated udder whereas 1 liter of prestimulation blood had no effect This observation was confirmed by Peeters *et al* (1947)

Andersson (1951) observed that electrical stimulation of the supra optic nucleus in lactating goats caused milk ejection He removed 50 ml of blood from the external jugular vein during such stimu

lation and injected it intravenously into a second lactating goat A definite ejection of milk resulted in the recipient

These few observations on the oxytocin content of blood are of considerable importance qualitatively in helping to establish the mechanisms of action of the hormone but they do not provide adequate quantitative estimates of physiological concentrations Apparently no estimates of oxytocin in human plasma have yet been made by wholly acceptable methods

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PART FOUR

Steroid Hormones

CHAPTER X

Androgens in Human Plasma

Claude J Migeon

ORIGIN OF THE ANDROGENS OF HUMAN PLASMA
- The testis
- The adrenal gland
- The ovary
- Androgens arising from the metabolism of corticosteroids

NATURE OF THE ANDROGENS DETECTED IN HUMAN PERIPHERAL PLASMA

METHODS FOR THE QUALITATIVE AND QUANTITATIVE MEASUREMENT OF PLASMA ANDROGENS
- Ethanol extraction
- Hydrolysis and extraction
- Removal of phenols and other acidic compounds
- Purification of the extracts
- Fractionation of the 17 ketosteroids
- Quantitative determination
  - Zimmermann reaction
  - Reaction of Dirscherl and Zilliken
  - Other color reactions
  - Ultraviolet studies

METABOLISM OF EXOGENOUS ANDROGENS IN HUMAN PLASMA

PLASMA ANDROGENS IN PHYSIOLOGICAL AND PATHOLOGICAL CONDITIONS
- Levels of peripheral plasma 17 ketosteroids in normal young adult males
- Levels of peripheral plasma 17 ketosteroids in normal young adult females
- Effects of aging
- Levels of peripheral plasma 17 ketosteroids in infants and children
- Effects of pregnancy
- Anencephaly
- Binding of androgens to human plasma proteins
- Effect of ACTH
- Effect of human chorionic gonadotropin
- Plasma 17 ketosteroids in anuria
- Syndrome of virilism

CONCLUSIONS

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Classically an androgen may be defined as a substance capable of stimulating male secondary sex characteristics (Dorfman and Shipley 1956) It must be pointed out that such substances also have effects on other systems of the body and possibly some of these effects may be of greater significance than the action on sexual structures

From the definition compounds exerting androgenic activity could have a variety of chemical structure However the physiologically occurring androgens are steroids Since compounds with 21 carbon atoms will be considered in other chapters of this book we will confine our discussion to the C_{19} steroids

ORIGIN OF THE ANDROGENS OF HUMAN PLASMA

Evaluation of the role of endocrine glands as androgen producers presents numerous difficulties The isolation of hormones from a gland may be misleading because some of the products identified may be only intermediaries in the synthesis of the final active compounds or even artifacts arising from the extraction procedures Moreover the glands usually store very small amounts of hormones The direct technique of determining glandular activity by the measurement of the hormone content of the efferent vein blood of the gland is often difficult while surgical trauma inherent in such procedure might influence glandular activity Although a rather large amount of data on isolation of androgens from the endocrine glands of various animals is available there is at present a paucity of information concerning man While the testis and the adrenal gland are considered to be the principal sites of androgen biosynthesis there is also some evidence *that the ovary is capable of producing C_{19} steroids*

The Testis

Testosterone has been isolated from the testes of several mammalians (David *et al* 1935 Tagmann *et al* 1946 Prelog *et al* 1947) It has been isolated also following incubation (Brady 1951 Rabinovitz 1956) and perfusion (Savard *et al* 1952) of human testes with C^{14} labeled acetate (Table I) Another compound Δ^4 androstene 3 17 dione has been isolated after perfusion of human testes (Savard *et al* 1952) Incubation studies of rat (Slaunwhite and Samuels 1956 Lynn and Brown 1956) and human (Savard *et al* 1956) testicular tissue indicate

TABLE I *In Vitro* Studies with Glandular Tissues in Physiological and Pathological States

A th	Exp mental	Sub t t	Resulting androg ns
A TESTICULAR TISSUE			
B ady (1951)	No mal Tis li Incub ti	C^{14} A tat	T toste e
S ard et l (19 2)	No mal P rf i	C^{14} A etat	Te to t r Δ^4 D ne
Rabino tz (1956)	Homog nate In ubati	C^{14} Ac tate	T to te
Sa d t l (1956)	Inte t ll l ell tum (5 7/12 y s) Ti e lic Inc b ti	C^{14} A tate	T to ier Δ^4 D 11β OH Δ^4 Di e Δ^4 Tri
		C^{14} P ge t o e	T to te e Δ^4 D o e 11β OH Δ^4 D
		C^{14} Chol t r l	Δ^5 Andro t nedi l Dehydr p andro te
Bagg tt et l (1957)	U d fle tl ted emb yologi al t m Tis li I bati	C^{14} Ac tate	Δ^4 Dio 11β OH Δ^4 Di ne
B ADRENAL TISSUE			
Bl h t al. (1955)	No mal (31 w ks g tati) d male p eudoh maphr d t (t t m) N bati	N	Δ^4 Dione
T h t e et al (19 5)	Ad lt (hyp tens o r m ta t t can) Incub t	None	11β-OH Δ^4 D e
Bloch et l. (1956)	N l f tu — 9 21 w ks g tat N nc bat	N	Δ^4 Dio 11β OH Δ^4 Dio Dehydro p a dro te
Bl h t aL (1957)	Ad g ital yndr m (28 y s) Tiss l Inc b ti	C^{14} Acetat	Δ^4 D e 11β OH Δ^4 Dione Dehydr p andr te
Planti et aL (1957)	V il l g adr nal ti al tumo No In bati	N	Dehydr piandr stero e
Solomo et l (1958)	N mal fetus Hom g ates In ub t n	C^{14} P ge t e	Δ^4 D o e
Bl h B i hk (1959)	N m l fetu 12 22 w eks ge tati n Tis sli s Incub ti n	C^{14} Ac tate	Δ^4 Dio e 11β OH Δ^4 Dione Dehydr epiandr ster
C OVARIAN TISSUE			
Sa ard t l. (1957)	Arrh blastoma Tissu l In b t	C^{14} I g ter e	Δ^4 Di ne T to t ne

that the sequence Δ^5 pregnen 3β ol 20 one$\rightarrow$progesterone$\rightarrow$17 hydroxy progesterone $\rightarrow$ Δ^4 androstene 3 17 dione and testosterone is an important route of biosynthesis of androgens by the testis (Fig 1) However

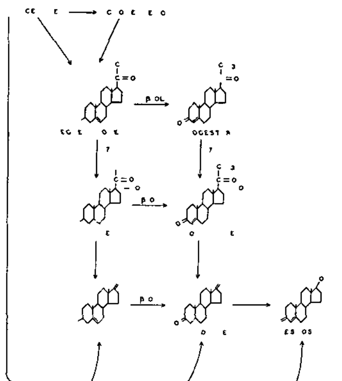


FIGURE 1 Possible pathways of biosynthesis of androgens by human gonads

other biosynthetic pathways may play a role in the production of androgens by the male sex gland As shown in Figure 1 C_{19} steroids could arise from Δ^5 pregnen 3β 17α diol 20-one in which case dehydro epiandrosterone (DHA) would be the precursor of Δ^4 androstene 3 17 dione and testosterone Moreover it is also possible that C_{19} steroids could come from acetate and/or cholesterol without 21 carbon atom intermediaries Finally certain tumors of the testis (Table I) have been found to produce steroids with an oxygen atom at carbon 11 (Baggett *et al* 1957 Savard *et al* 1957) these findings are rather surprising in view of the fact that it has been generally assumed that the testis normally or pathologically could produce only 11 deoxy C_{10} steroids

Testosterone and Δ^4 androstene 3 17 dione have been isolated from spermatic vein blood of dogs (West *et al* 1952) More recently the former compound was identified in the extract of a large pool of

spermatic vein blood from 8 patients with prostatic cancer (Lucas *et al* 1957) the testosterone concentration was approximately 1 0 μg per hundred milliliters of whole blood The subjects in this study were elderly men a fact which might explain the low hormonal concentrations reported It has since been shown that younger men can have concentrations of testosterone up to 160 μg per hundred milliliters of spermatic vein plasma (Hollander and Hollander 1958) these levels decrease progressively with age (Fig 2)

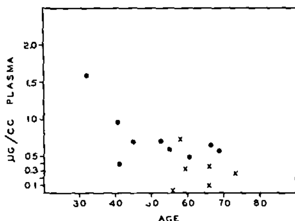


FIGURE 2 Concentration of testosterone in human spermatic vein blood (in micrograms per milliliter of plasma) The black circles represent normal controls the crosses represent patients with carcinoma of the prostate (From Hollander N and Hollander V P *J Clin Endocrinol and Metabolism* 18 966 1958)

The Adrenal Gland

Although Δ^4 androstene 3 17-dione (von Euw and Reichstein 1941) its 11 ketone derivative (Reichstein 1936) and androstane 3β 11β diol 17 one (Reichstein and von Euw 1938) have been isolated from beef adrenal glands there is no report concerning the isolation of C_{19} steroids from normal adult human adrenal glands However Touchstone *et al* (1955) have isolated 11β hydroxy Δ^4 androstene 3 17 dione from adrenal incubates of patients with either hypertension or metastatic cancer In addition to this latter compound Bloch *et al* (1955 1956) have also detected Δ^4 androstene 3 17 dione and dehydro epiandrosterone in fetal adrenal glands (see Table I) The same three C_{19} steroids have been identified following incubation with C^{14} acetate of adrenal gland tissue from a patient with adrenogenital syndrome

(Bloch *et al* 1957) and from normal human fetuses (Bloch and Benirschke 1959) while homogenates of human fetal adrenals incubated with C^{14} progesterone gave rise to Δ^4 androstene 3 17 dione (Solomon *et al* 1958) Plantin *et al* (1957) also isolated dehydroepiandrosterone from the virilizing adrenal tumor of a 62 year old female

The androgens detected in adrenal vein blood are listed in Table II Two hormones have been chemically isolated i e 11β hydroxy Δ^4

TABLE II Androgen Content of the Blood from the Efferent Vein of Testis Ovary and Adrenal Gland

So	Auth	Expe im nt l bject	C mpounds	Co c nt ti
Sp rmatic ein blood	L c s et al (1957)	8 S bjects (50 74 yrs) — pool Prostatic cancer	Testo te one	App 1 µg /100 ml blood
	Holl nder a d H ll de (1958)	20 S bje t (30 78 yrs) — 10 p tatic cancer — 10 mal	Testoste one	160 2 5 µg /100 ml plasma
Ad e al ei blood	Roma off et al (1953)	1 Metastatic pr static ca r	11β OH Δ^4 di ne	84 534 µg /100 ml blood
	Pi cus and Romanoff (1955)	1 M tastatic br ast ca cer — Rx ACTH	Δ^4 Dio e	32 39 µg /100 ml blood
	B sh et al (1956)	Post p bertal v ilism — Rx ACTH	11β OH Δ^4 d o e DHA† Androst rone	93 µg /100 ml blood 93 µg /100 ml blood 40 µg /100 ml bl od
	Migeon, Lescure (npubli h d)	Cush g's syndrome (bilate al adre al hyp plasia)	11β OH Δ^4 dione Δ^4 Dione	280 µg /100 ml pl m 70 µg /100 ml plasma
		Adre al rest tumor	Δ^4 Dio e Etiocholanolone	45 µg /100 ml plasma 30 µg /100 ml plasm
Ovarian vei blood	M g on et al (1957)	Stei Leventhal syndr m	Δ^4 Di Andr stero e	29 µg /100 ml pl sma 36 µg /100 ml plasma
		Ar henobl stoma	Δ^4 Dio e Androstero e Etiocholanolone	24 µg /100 ml pl sma 10( ) µg /100 ml plasma 8(±) µg /100 ml pla ma

Thes t roids h b h mi ally i l ted
† Dehydroep andro tero

androstene 3 17 dione and Δ^4 androstene 3 17 dione (Romanoff *et al*, 1953 Pincus and Romanoff 1955) The same compounds have been detected in a case of Cushing s syndrome due to bilateral adrenal hyperplasia (Migeon and Lescure) while two additional steroids — dehydroepiandrosterone and androsterone — were found in a case of postpubertal virilism (Bush *et al* 1956) Figure 3 shows the various possible pathways for the biosynthesis of androgens by the adrenal gland

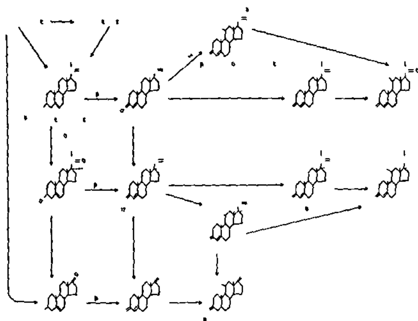


FIGURE 3 Possible pathways of androgen biosynthesis by the human adrenal cortex

The Ovary

Although C_{19} steroids have not been isolated from ovarian tissues up to the present there is some evidence that the ovary can produce such compounds (Parkes 1950) Extracts prepared from this gland have shown the presence of androgenic activity (Champy and Kritch 1925 Parkes 1937)

It has been pointed out that ovariectomy in the human being is not followed by the decline in urinary 17 ketosteroids which characterizes ablation of either the testes or the adrenal glands (Dorfman and Shipley 1956) Although the ovary is probably not a major source of androgens in normal women three conditions with ovarian abnormalities are known to produce marked virilization arrhenoblastoma adrenal rest tumor of the ovary and Stein Leventhal syndrome (polycystic ovaries)

In a case of arrhenoblastoma incubation of the tumor with C^{14} progesterone has demonstrated the conversion of this C_{21} steroid into small amounts of Δ^4 androstene 3 17-dione testosterone and possibly 11β OH Δ^4 androstene 3 17-dione (Savard *et al* 1957) A similar tumor incubated with progesterone-4 C^{14} has been reported to yield

Δ^4 androstene 3 17 dione and 17α hydroxyprogesterone (Wiest *et al* 1959) Homogenized bovine ovarian tissue incubated with progesterone C^{14} has been shown to produce 17α hydroxyprogesterone and Δ^4 androstene 3 17 dione (Solomon *et al* 1956)

In a case of adrenal rest tumor of the ovary Migeon *et al* (1957) have identified Δ^4 androstene 3 17 dione and etiocholanolone in the extract of the blood from the vein of the tumor The same authors have detected Δ^4 androstene 3 17 dione and androsterone in ovarian vein blood from a patient with arrhenoblastoma and one with Stein Leventhal syndrome (see Table II)

Although there is no direct evidence that the normal human ovary produces androgens the observations reported above suggest that this gland has the potentiality to do so and that in pathological cases it certainly can synthetize C_{19} steroids

Androgens Arising from the Metabolism of Corticosteroids

During their metabolism in the body corticosteroids lead to the production of C_{19} steroids On the basis of evidence presently available it seems that only the corticosteroids with a 17 hydroxyl group can be transformed into C_{19} androgens Furthermore it has been suggested that active corticosteroid hormones which possess a Δ^4 3 ketone grouping are first metabolized into a C_{21} saturated intermediate prior to losing their side chain most of the C_{21} saturated metabolites having a 5β configuration the corresponding C_{19} steroids will be mostly the 5β stereoisomers At the same time the metabolism of Δ^4 3 keto C_{19} steroids to saturated C_{19} steroids gives significantly less 5β than 5α isomers if the precursor has an oxygen atom on carbon 11 and approximately equal amounts of 5β and 5α isomers if the precursor does not possess an oxygen in the 11 position

On the basis of such considerations calculations of the actual production of various steroids have been made (Dorfman and Ungar 1953 Dorfman 1954 Dorfmann and Shipley 1956) Such calculations however are more theoretical than factual and must at present be considered as interesting speculations Some objections have been formulated mainly on the ground that steroid metabolism is a dynamic process and that it has been shown that the ratio of 5β to 5α isomers may shift with the time following infusion of a test compound (Gallagher *et al* 1954) This ratio can also be altered by changes in the physiological status of the experimental subject (Bradlow *et al* 1956)

Finally it is generally admitted that if the C_{21} steroid precursor

possesses an oxygen atom at carbon 11 the C_{19} steroid derivative will also have an 11 ketone or 11 hydroxyl group Consequently 11-oxy C_{19} steroids can be considered of adrenal origin either directly or indirectly however the reciprocal is not necessarily true a large amount of 11-desoxy C_{19} steroids being produced by the adrenal gland

In summary it appears that the human testis secretes testosterone Δ^4 androstene 3 17 dione and perhaps in certain pathological conditions some 11-oxygenated-C_{19} steroids while the human adrenal gland secretes 11β hydroxy Δ^4 androstene 3 17-dione Δ^4 androstene 3 17-dione and dehydroepiandrosterone (DHA) although it is certain that ovarian tumors can produce androgens it is difficult at present to ascertain the nature as well as the quantitative importance of C_{19} steroid secretion by the normal human ovary

NATURE OF THE ANDROGENS DETECTED IN HUMAN PERIPHERAL PLASMA

Table III shows the C_{19} steroids which have been detected in peripheral human plasma

Dehydroepiandrosterone and androsterone have been chemically isolated (Migeon and Plager 1954 Clayton *et al* 1955 Migeon 1956a) Present evidence strongly suggests that these two steroids are present in peripheral human plasma as sulfates since maximum yields are obtained when a 48 hour continuous ether extraction at pH 0 8 is used (Migeon and Plager 1955) Furthermore the sulfates of dehydro epiandrosterone and androsterone appear to be the major C_{19} 17 ketosteroids present in peripheral blood of man

Until recently androgen sulfates have been the only 17 ketosteroids detected in human peripheral blood apparently because these com pounds are the major components of blood androgens However using large plasma samples (1500 to 2000 ml) it has been possible to detect small amounts of free steroids such as dehydroepiandrosterone an drosterone Δ^4 androstene 3 17-dione and its 11β hydroxy derivative (Table III) In smaller plasma samples of approximately 250 ml Kellie and Smith (1957) were not able to detect any free androgens The most probable explanation for the high levels of free plasma 17 ketosteroids reported by Tamm *et al* (1957 1958b) is that these authors were measuring large amounts of nonspecific chromogens It must be noted that testosterone was not detected either in 300 ml or in 2500 ml pools of peripheral plasma (Migeon 1955 1956b) Since

TABLE III Concentiations of 17 Ketosteroids of Human Peripheral Plasma

Ide tif ed 17 k to tero d	A th s	Males			F mal		
		Rang	M an	N C	Rang	Mean	N cas s
A. FREE PLASMA 17 KETOSTEROIDS							
Dehydroepiandro terone	Mig on (1957)	0 02 04	—	3	0 02	—	2
Andro terone	Migeon (1957)	0 0 02	—	3	0 0	—	2
Δ^4 D o e	Migeo (1957)	0 0 025	—	3	0 03	—	2
11β ol Δ^4 Di ne	Tamm et al (1958b)‡						
B SULFATE PLASMA 17 KETOSTEROIDS							
D hydroep and osteron †	Migeon and Plager (1954) M geon t al (1957) Cl yton et al (1955) Kellie a d Smith (1957) Tamm t l (1958b)‡	20 to 85 — 12 to 131	48 2 76 47	24 4 6	8 1 to 81 4 7 to 46	39 4 22 5	16 5
And o te o †	Clayto et l (1955) Mig o (1956a) M g et al (1957) Kell e a d Sm th (1957) Tamm et al (1958b)‡	— 9 to 52 1 3 to 43	13 0 25 0 15	4 24 6	0 to 48 5 1 to 27	19 8 13	16 5
II K to t o holanol e	Tamm et al (1958b)‡						
C GLUCURONOSIDE PLASMA 17 KETOSTEROIDS							
And oste one	Kell d Smith (1957) M g o (1957) Tamm t l (1958b)‡	2 1 1 5 1 2 0 8		1 3	1 5 2 0 8 1 3		2 2
Etio h lanolo	Kelli a d Sm th (1957) M geon (1957) T mm et al (1958b)‡	1 3 0 6 1 0 1 5		1 3	1 2 5 1 1 1 4		2 2
11 Keto t chola l e	Kell d S ith (1957) Mige (19 7) T m et al (1958b)‡	— 0 02 0 75		3	0 3 0 035		1 2
11β OH A dro te e	Kell e and Sm th (1957)	0 9		1			
De hydro p d t ne	K ll e and Sm t (1957) Tam et al (1958b)‡	2 8		1	0 17		2
D STRONG ACID HYDROLYSIS PLASMA 17 KETOSTEROIDS							
And ost	Ta m et l (1958b)‡						

\ l μg /100 ml
† Th t d h b h mically l ted
‡ N l ilabl

the smallest amount of this steroid detectable on paper chromatograms is 5 μg it would appear that testosterone concentration in peripheral plasma of man is less than 0 2 μg per hundred milliliters Even the more sensitive method of Hollander and Hollander (1958) could not demonstrate the presence of testosterone in peripheral blood

In addition the following androgens have been detected following β glucuronidase hydrolysis androsterone etiocholanolone 11 keto

etiocholanolone and 11β hydroxyandrosterone The concentration of each of these androgens varies between 0 3 and 2 5 μg per hundred milliliters of plasma as shown by Kellie and Smith (1957) and Migeon (1957) Tamm *et al* (1957) have reported a mean value of 68 6 μg per hundred milliliters for the total 17 ketosteroids of the glucuronoside fraction this level appears to be much overestimated when compared to the results of the other investigators mentioned above

Kellie and Smith (1957) have identified small amounts of dehydro epiandrosterone in the glucuronoside fraction of two of their large plasma samples (1 7 and 2 8 μg per hundred milliliters) despite inhi bition of the sulfatase activity of their enzyme preparations by 0 03 M KH_2PO_4 while Tamm *et al* (1957) have reported high levels of dehydroepiandrosterone in this same fraction

As can be seen a great number of androgens have been detected in human peripheral plasma it must be stressed however that at present only two androgens have been thoroughly isolated and identified by means of infrared spectrum and melting point These two compounds are dehydroepiandrosterone and androsterone which are probably present in plasma as sulfate conjugates It is our opinion that these are the major components of the total plasma 17 ketosteroids The predominance of sulfate conjugates over the glucuronoside conjugates as well as the very small concentration of free androgens is discussed under the metabolism of androgens in man (p 315)

METHODS FOR THE QUALITATIVE AND QUANTITATIVE MEASUREMENT OF PLASMA ANDROGENS

The first attempts to measure androgenic hormones from human blood were made by McCullagh and co workers (1933) using capon comb growth as a test of androgenicity It was observed that ether extracts of 50 ml of blood from normal men were able to stimulate capon comb growth Somewhat similar results were reported by Koch (1937–1938) Tornblom (1946) has also carried out extensive studies of androgen activity in human blood Methods of androgen bioassay have been reviewed and discussed by Dorfman and Shipley (1956) and Loraine (1958)

The biological determination of plasma androgens is of great theoretical interest It is generally admitted that the free hormones of blood are the physiologically active compounds while the conjugated

hormones are inactive products of catabolism The extraction of free androgens from blood and the determination of the biological activity of this extract appear therefore to be a direct measurement of target organ androgenicity However such techniques have limitations in herent in all biological assay one of the major objections is that the responsiveness of the target organ of the test animal to a given com pound might not correspond to the androgenicity of this compound in the human subject For this reason efforts have been made to develop chemical methods of measurement of plasma androgens

Zimmermann (1944) published a technique for the measurement of 17 ketosteroids in serum No special attempt was made to eliminate the large amount of fat extracted along with the steroids and the values given by this procedure were very high This author stated (1946) that it was difficult to evaluate the technique Dumazert and Valensi (1952) described another method after sulfuric acid hydroly sis the plasma is desiccated by anhydrous sodium sulfate and the heavy pasty sludge is then extracted with ether The plasma concen trations of 17 ketosteroids obtained by this method are almost as high as the average values usually found for urinary 17 ketosteroids

West *et al* (1951) have studied the plasma 17 ketosteroids resulting from the metabolism of testosterone administered intravenously to normal human beings Their method consisted of the following steps ethanol precipitation of plasma proteins hydrolysis with hydrochloric acid extraction of the hydrolyzate with ether washing of the extract with alkali acid and water and chromatography on alumina column However using the analytical procedure described these authors were not able to detect androgens in the blood of normal adult males

There are only a small number of reports on analytical methods for the measurement of plasma androgens in normal individuals Gardner (1953) has described a technique of estimation of total neutral 17 ketos teroids while Migeon and Plager (1955) Clayton *et al* (1955) Kellie and Smith (1957) and Tamm *et al* (1958b) have described methods of fractionation of these steroids

The method described in the following outline would in the author s opinion meet the requirements for a reliable study of 17 ketosteroids of peripheral plasma

(1) *Ethanol extraction*

1 v plasma + 5 v 100% EtOH Stir centrifuge remove EtOH extract

Wash protein residue twice with 1 v 80% EtOH Centrifuge add EtOH to first extract

Evaporate EtOH Take residue in 1 v distilled water

(2) *Hydrolysis and extraction*

(a) *Free fraction*

Extract aqueous residue with 2 v redistilled $CHCl_3$ or $CH_2Cl_2 \times 3$

(b) *Glucuronoside fraction*

To aqueous residue add 0 1 v 2 M acetate buffer and 0 1 v β glucuronidase (Ketodase)

Adjust pH to 4 6

Incubate at 37°C for 72 hours

Extract aqueous residue with 2 5 v redistilled $CHCl_3$ or $CH_2Cl_2 \times 3$

(c) *Sulfate fraction (continuous ether extraction)*

Adjust pH of aqueous residue to 0 8 with H_2SO_4

Extract continuously with ether for 48 hours

(d) *Strong acid hydrolysis*

To the aqueous residue add 0 1 v of concentrated H_2SO_4

Put in boiling water bath for 30 minutes

Cool

Extract with 3 v redistilled $CHCl_3$ or $CH_2Cl_2 \times 3$

From there on each of these fractions is treated separately

(3) *Removal of phenols and other acidic compounds*

Reduce volume of the extracts to approximately 50 ml

Wash with 1 N NaOH (10 ml $\times$ 2) and water (20 ml $\times$ 3)

Evaporate organic phase to dryness

(4) *Purification of the extracts*

(a) *Solvent partition*

Dissolve dry residues in n hexane (10 ml $\times$ 2) and pour into separatory funnel

Extract n hexane with 70% EtOH (20 ml $\times$ 1 and 15 ml $\times$ 2)

Add 50 ml distilled water to pooled 70% EtOH extracts

Extract aqueous EtOH with $CHCl_3$ (50 ml $\times$ 3)

(b) *Florisil column chromatography*

Transfer residues on column with exactly 20 ml $CHCl_3$

Extract 17 ketosteroids by elution with 35 ml of 2% EtOH in $CHCl_3$

(5) *Fractionation of the 17-ketosteroids*
 (a) Paper chromatography
 (b) Column chromatography
(6) *Quantitative determination*
 (a) Zimmermann reaction
 (b) Reaction of Dirscherl and Zilliken
 (c) Other color reactions
 (d) Ultraviolet studies

(1) Ethanol Extraction

Free steroids can be extracted directly by chloroform or methylene chloride. However, following ethanol extraction, β-glucuronidase hydrolysis and continuous ether extraction at pH 0.8 are greatly facilitated and are certainly more reliable when the plasma proteins have been precipitated. Since ethanol extraction must be carried out at one time or another, it is probably better to do it at the beginning (West *et al.*, 1951; Clayton *et al.*, 1955; Migeon and Plager, 1955; Kellie and Smith, 1957; Tamm *et al.*, 1957).

(2) Hydrolysis and Extraction

It is certainly important to determine the concentrations of free androgens *in peripheral plasma since these are* the levels of active hormone expected to be present in the target organs. It is also of interest to know about the nature and the concentration of the conjugated compounds. If a relatively small amount of plasma (15 to 30 ml) is used for the assay, it is impossible to detect either free or glucuronoside steroids. In normal individuals at least 50 ml of plasma is necessary to detect the androgens liberated by β-glucuronidase hydrolysis, and significantly larger amounts of plasma are needed to visualize free androgens.

It is our experience that chloroform, methylene chloride or benzene will extract quantitatively androgens from aqueous solution by the method indicated in the outline, and therefore any one of these solvents is satisfactory for extraction.

The techniques of β-glucuronidase hydrolysis, continuous ether extraction at pH 0.8 and strong acid hydrolysis are classic. Recently Burstein and Lieberman (1958a) have devised two new procedures for the hydrolysis of urinary steroid sulfates. In these methods urine is brought to an acid concentration of 2N with 50% H_2SO_4 and an equal volume of either ethyl ether or ethyl acetate is added. The ether

and urine are vigorously shaken 5 times daily for 4 days after which time the ether phase is separated The ethyl acetate and urine are mixed thoroughly once and the ethyl acetate extract is separated from the aqueous phase and allowed to stand at 38°C for 24 hours The same authors have also studied the kinetics and mechanism of the solvolysis of steroid sulfates (Burstein and Lieberman 1958b) If the β glucuronidase preparation contains some sulfate activity it might be well to inhibit this activity by 0 03 M KH_2PO_4 (Roy 1956) as indicated by Kellie and Smith (1957)

(3) Removal of Phenols and Other Acidic Compounds

The sodium hydroxide washing is followed by a washing of the extract with water until neutral

(4) Purification of the Extracts

Depending upon the amount of plasma used more or less extensive purification of the extracts must be carried out

Solvent partition can certainly remove a large amount of unwanted fatty material A technique studied by Samuels (1947) and described in the outline is satisfactory Gardner (1953) uses a slightly modified method Tamm *et al* (1958a) prefer the use of a solvent somewhat similar to skellysolve B rather than n hexane as they claim to have some difficulties with the latter solvent Slaunwhite and Sandberg (1957) have utilized the systems n hexane 70 per cent methanol and 45 per cent methanol benzene

Florisil column is another efficient means of purification of androgen extracts The Florisil is prepared as described by Eik Nes *et al* (1953) Gardner (1953) extracts the 17 ketosteroids from the column with 47 ml of chloroform It is our experience (Migeon and Plager 1955) that the first 20 ml of chloroform does not extract androsterone or dehydroepiandrosterone and therefore this first fraction is discarded while the next 35 ml of 2 per cent methanol (or ethanol) in chloroform which contains the 17 ketosteroids is saved It must be noted that androsterone and dehydroepiandrosterone can be obtained by elution by chloroform only The 2 per cent methanol in chloroform is used in order to be sure that the more polar androgens (11 keto and 11 hydroxy 17 ketosteroids) are extracted

It is evident that solvent partition and Florisil column chromatography can be carried out simultaneously in order to attain greater purification of the extracts

(5) Fractionation of the 17 Ketosteroids

A fractionation of urinary 17 ketosteroids with alumina column was first described by Dingemanse *et al* (1946) Lakshmanan and Lieberman (1954) applied to that method the gradient elution principle and achieved greatly improved separation of the individual steroids Kellie and Smith (1957) have successfully adapted the fractionation on alumina column with gradient elution to the separation of plasma 17 ketosteroids of man Slaunwhite and Sandberg (1957) have used the technique of Lakshmanan and Lieberman with success The inability of Clayton *et al* (1955) to separate plasma 17 ketosteroids might be related to the large amount of material applied to the alumina column

Since 1949 paper chromatography has been widely used as a means of separation of steroids Two main techniques each with several variants are currently employed in the first method the paper is impregnated with nonvolatile organic solvents (Zaffaroni *et al* 1949) in the second method the paper is untreated (Bush 1952) In addition Kritchevsky and Tiselius (1951) have described a reverse phase technique The relative merits of the various techniques of paper chromatography and for that matter of paper chromatography over column chromatography in steroid studies have been the subject of a great deal of controversy Details of these controversial issues may be found in *Recent Progress in Hormone Research,* Volume 9 (especially the papers and ensuing discussion of Savard K Rubin B L *et al* and Bush I E)

A comprehensive review of the various paper chromatographic methods for steroid fractionation has been made by Block *et al* (1955) Tamm *et al* (1958b) utilize successively the systems of solvents of Kritchevsky and Tiselius (1951) Kochakian and Stidworthy (1952) and Pechet (1955) while Slaunwhite and Sandberg (1957) identify the steroids separated by column chromatography by the paper chromatographic technique of Bush (1952) We use also the latter method Table IV shows the R_f values of a certain number of C_{19} steroids in the systems of solvents skellysolve C benzene methanol water (66 33 80 20) and skellysolve C methanol water (100 85 15) These R_f values are relative rather than absolute small variations in experimental conditions producing notable changes for example in the system skellysolve C methanol water a slight decrease in the proportion of methanol markedly increases the R_f

TABLE IV R_f Values of C_{19} Steroids at 37 C Using Whatman No 2 and Bush Systems of Solvents

C_{19} = Steroids	Skelly C Methanol Water (100 85 15)	Skelly C Benzene Methanol Water (66 33 80 20)
11β Hydroxy etiocholanolone	0	0 035
11α-Hydroxy Δ^4 androstene 3 17 dione	0	0 105
11β Hydroxy-Δ^4 androstene 3 17 dione	0 019	0 299
11 Keto etiocholanolone	0 024	0 358
11 Keto Δ^4 androstene 3 17 dione	0 044	0 447
Testosterone	0 080	0 466
11 Keto androstane 3 17 dione	0 160	0 585
Dehydroepiandrosterone	0 162	0 590
Epiandrosterone	0 190	0 633
Etiocholan 3α ol 17 one	0 205	0 640
Etiocholan 17-ol 3 one	0 242	0 700
Δ^4-Androstene 3 17 dione	0 242	0 703
Δ^1 Androstene-3 17 dione	—	0 750
Androsterone	0 310	0 757
Etiocholane 3 17 dione	0 397	0 780
Androstane 3 17 dione	0 402	0 790

values The relative merits of the various systems of solvents and techniques available seem to be often determined by individual preference In most cases a minimal modification of a system will give a method adaptable to the needs of any investigation

(6) Quantitative Determination

Zimmermann Reaction The Zimmermann reaction (1936) is the most widely used method for the measurement of 17 ketosteroids Kritchevsky and Tiselius (1951) adapted the method to the detection of spots on paper chromatograms

The eluates of column chromatography can be used directly for quantitative determination In paper chromatography an aliquot of

the extract must be run separately and the spots must be detected on the papergram the area of the papergram of the rest of the extract corresponding to the purple spots of the aliquot is carefully cut out and the steroid is extracted by ethanol or methanol

In any case the amount of steroid present in the eluates being very small micro adaptation of the Zimmermann reaction is necessary This is done by decreasing the volumes of ethanolic meta dinitrobenzene and potassium hydroxide for the reaction itself and also by decreasing the amount of ethanol used for the dilution of the final product of the reaction (Gardner 1953 Tamm *et al* 1957 Kellie and Smith 1957) Bongiovanni and Eberlein (1957) have applied the micromethod of Wilson (1954) the reaction is carried out at 0°C because the development of the nonspecific brown color is selectively inhibited at this temperature while the purple color of 17 ketosteroids becomes well developed if sufficient time is allowed

In the technique of Holtorff and Koch (1940) 5 normal aqueous potassium hydroxide replaces the 2 5 normal ethanolic potassium hydroxide Cahen and Salter (1944) have observed that an organic solvent such as chloroform or ether can extract the purple chromogens formed by the 17 ketosteroids during the reaction without extracting the nonspecific brown color However it has been pointed out that aqueous potassium hydroxide may give unreliable results For this reason Migeon and Plager (1954) have devised the following modification the color reaction is developed with ethanolic potassium hydroxide but then water is added and the purple chromogens are extracted with chloroform Unfortunately the Δ^4 3 ketones cause a very cloudy reaction which interferes with the reading

Most investigators apply the correction formula of Allen (1950) in order to compensate for the nonketonic chromogens

Reaction of Dirscherl and Zilliken (1943) This reaction is *relatively specific for dehydroepiandrosterone* and some of its derivatives and has been the basis for several modifications (Patterson 1947 Jensen 1950 Allen *et al* 1950) which can be applied to the measurement of dehydroepiandrosterone in human plasma (Bongiovanni and Eberlein 1957)

Other Color Reactions The Pettenkofer reaction has been adapted to the measurement of dehydroepiandrosterone (Munson *et al* 1948) this method however is not specific for this steroid

The Pincus reaction (1943) has also been applied to the measurement of 17 ketosteroid in plasma (Bongiovanni and Eberlein 1957)

The presence of nonketonic steroids has been detected by spraying 50 ml of the phosphomolybdic reagent on papergrams (Kochakian and Stidworthy 1952)

Ultraviolet Studies $\alpha\beta$ Unsaturated ketosteroids developed on paper can be visualized by a fluorescence scanner (Haines and Drake 1950) The fluorescence in ultraviolet light following treatment with methanolic sodium hydroxide (Bush 1952) has been found specific for compounds with a Δ^4 3 ketone in their molecule Each of these techniques is of great value in characterizing steroids on papergrams and could be applied to the quantitative analysis of these steroids

The absorption spectra of sulfuric acid chromogens is used in the identification of steroid eluates from either column or paper chromatography (Zaffaroni and Burton 1951)

Since the requirements essential to a good hormone assay are not thoroughly met by the techniques presently available the methods of measurement of plasma androgens in man are still unsatisfactory

In order to detect the minute amounts of free androgens one must work with a very large plasma sample At least 50 ml of plasma are necessary for the detection of the glucuronosides and only somewhat less for the sulfates The color reactions used are not very specific and the use of large plasma samples increases the amount of nonspecific compounds in the extracts In turn the extensive purification which must be carried out decreases the precision of assay Also it must be added that the R_f value of a spot or the number of the peak tube of a column fractionation is not sufficient to fully identify a steroid especially in some pathological conditions where spot checks for the identity of the compounds encountered have never been made

The micromethods devised for the measurement of 17 ketosteroids enable the investigator to work with smaller amounts of material however such methods decrease necessarily the precision of the assay

METABOLISM OF EXOGENOUS ANDROGENS IN HUMAN PLASMA

The study of the metabolism of exogenous androgens in human plasma is of great interest since it sheds some light upon the physiological disposition of these steroids and in addition contributes to the understanding of normal endogenous 17 ketosteroid metabolism in man

The first investigation of this type was that of West and collabora tors (1951) Testosterone (approximately 150 mg) dissolved in human serum albumin was given intravenously over a 10 to 20 minute period and blood samples were drawn periodically thereafter The free steroids were extracted from the blood with ether after purification of the extract quantitative analyses were made for $\alpha\beta$ unsaturated ketosteroids and 17 ketosteroids The blood residues were then sub mitted to hydrochloric acid hydrolysis as described previously Five minutes following the injection only 7 5 per cent of the expected concentration of testosterone could be demonstrated as $\alpha\beta$ unsaturated ketosteroids Subsequently the decline of testosterone concentration followed a logarithmic course In the free fraction 17 ketosteroids could not be detected yet they were found in large amounts in the con jugate fraction *with maximum concentration 20 minutes following* testosterone infusion

More recently methods have been developed for the synthesis of labeled testosterone and it has become possible to study the metabo lism of this hormone administered in physiological doses Sandberg and Slaunwhite (1956) have administered intravenously 1 to 5 micro curies of testosterone 4 C^{14} to normal individuals and have confirmed the results of West *et al* (1951) They have shown that testosterone disappears from the circulation very rapidly with at least two different rates and that the conjugated metabolites of testosterone reach their peak in plasma within 15 minutes (Fig 4) In addition these authors observed that while 12 to 14 per cent of the injected compound was present in the bile of patients with T tube drainage only 6 per cent was excreted in the stools of normal individuals suggesting a partial reabsorption of the steroids from the intestine The remaining radio activity was recovered in the urine in 48 hours with over 50 per cent of the administered dose recovered in the first 4 hours

Slaunwhite and Sandberg (1958) have studied also the metabolism of androsterone C^{14} and etiocholanolone C^{14} The free steroids were cleared from plasma even more rapidly than testosterone (Fig 4) It is of interest that the glucuronosides of etiocholanolone were removed from the circulation faster than those of androsterone Negligible amounts of hormone were recovered in the bile of bile fistula patients and the radioactivity was recovered in the urine almost quantitatively in a few hours

The compound 4 C^{14} Δ^4 androstene 11β-ol 3 17-dione has been found to have a rate of disappearance from the circulation inter

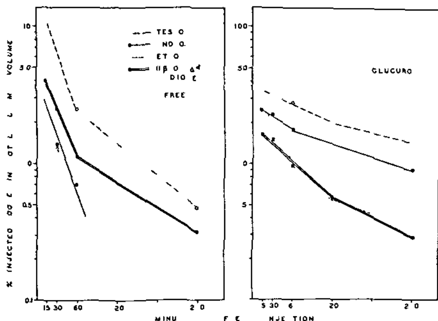


FIGURE 4 Radioactivity in the free and glucuronoside fractions of the plasma of subjects who received testosterone 4 C14 11β hydroxy Δ4 androstene 3 17 di one C14 androsterone C14 and etiocholanolone C14 The plasma volume was calculated as 5 per cent of the body weight (From Slaunwhite W R Jr and Sandberg A A *J Clin Endocrinol and Metabolism* 18 1056 1958 and from Sandberg A A and Slaunwhite W R Jr *Proc Soc Exp Biol Med* 96 658 1957)

mediate between testosterone on the one hand and androsterone and etiocholanolone on the other (Sandberg and Slaunwhite 1957) The glucuronoside fraction of this steroid was removed from plasma at least as fast as that of etiocholanolone (Fig 4) Similar results have been reported by Bradlow and Gallagher (1957) In reference to androsterone and etiocholanolone only small amounts of radioactivity were found in the bile while 95 per cent of the administered dose was recovered in the urine

The rapid disappearance from the circulation of exogenous tes tosterone might explain why testosterone has been isolated from spermatic vein blood yet cannot be detected in peripheral plasma Exogenous androsterone etiocholanolone and 11β hydroxy Δ4 androstene 3 17-dione are cleared even more rapidly than testosterone and it is therefore not surprising to find extremely low concentrations of free androgens in the peripheral plasma of normal men

Bongiovanni and Eberlein (1957) have studied the renal clearance of 17 ketosteroids in man Dehydroepiandrosterone acetate and andros

terone hemisuccinate were administered separately by mouth The renal clearances were calculated as

$$\frac{\text{urine steroids } (\mu g \text{ excreted per min})}{\text{plasma steroids } (\mu g \text{ per } 100 \text{ ml})} = \text{ml plasma cleared of steroid per min}$$

The renal clearance of androsterone was found to be much greater than that of dehydroepiandrosterone Furthermore the renal clearances of androsterone maximal at low plasma concentrations decreased with increasing plasma concentrations The clearances of dehydroepiandrosterone however appeared to be independent of the blood concentrations (Fig 5) From these and other experiments the

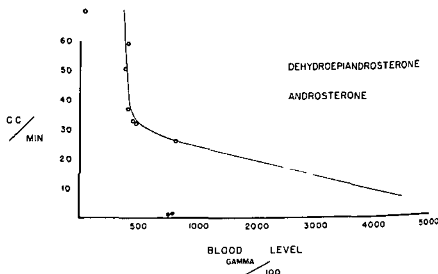


FIGURE 5 Results of all experiments relating blood levels of steroid (abscissa) to plasma clearance The curve for androsterone resembles that of the clearance of substances which are secreted by tubular mechanisms rather than glomerular filtration (From Bongiovanni A M and Eberlein W R *J Clin Endocrinol and Metabolism* 17 238 1957)

authors suggested that androsterone is eliminated mainly by tubular secretion in contrast with dehydroepiandrosterone which is excreted by glomerular filtration with little or no tubular reabsorption

Kellie and Smith (1957) from similar studies have come to the same general conclusions They measured the physiological concentrations of the sulfate and glucuronoside conjugates of plasma and urinary androgens and calculated that the renal clearance values for

dehydroepiandrosterone sulfate ranged from 0 5 to 6 0 ml per minute while those for androsterone sulfate ranged from 1 to 12 ml per minute The corresponding values for androsterone and etiocholanolone glucuronosides were 113 to 177 ml per minute

In another study 240 mg of testosterone dissolved in 100 ml of 25 per cent human serum albumin solution was administered intravenously over a 30 minute period to normal subjects Blood samples were collected at various times after the infusion and the plasma was analyzed for the 17 ketosteroids which had been liberated by β glucuronidase hydrolysis and continuous ether extraction at pH 0 8 The concentrations of androsterone and etiocholanolone freed by β glucuronidase hydrolysis were observed to be lower than those freed

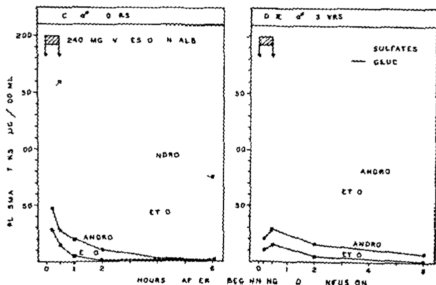


FIGURE 6 Plasma concentrations of androsterone and etiocholanolone (glucuronosides and sulfates) following the intravenous infusion of 210 mg of testosterone (From Migeon C J *et al J Clin Endocrinol and Metabolism* 17 1051 1957)

by continuous ether hydrolysis at pH 0 8 (Fig 6) Urine studies showed that approximately 30 per cent of the administered testosterone was excreted as 17 ketosteroids Eighty to 88 per cent of these total urinary 17 ketosteroids were found in the fraction freed by β glucuronidase hydrolysis with 5 to 9 per cent in the fraction produced by pH 0 8 hydrolysis

These studies help one to understand the physiological preponder

ance of androgen sulfates — especially dehydroepiandrosterone — in *human peripheral plasma* while in urine the glucuronosides make up the greatest part of the conjugated 17 ketosteroids

PLASMA ANDROGENS IN PHYSIOLOGICAL AND PATHOLOGICAL CONDITIONS

Levels of Peripheral Plasma 17 Ketosteroids in *Normal Young Adult Males*

The total plasma 17 ketosteroids have been reported to vary between 40 and 130 μg per hundred milliliters for men and in women between 25 and 100 μg per hundred milliliters (Gardner 1953)

The values for the individual 17 ketosteroids are summarized in Table III As noted earlier in this chapter the concentration of free 17 ketosteroids is very low

In 24 normal males 20 to 29 years of age the average concentration of dehydroepiandrosterone (sulfate) was 48 2 μg per hundred milliliters with a standard deviation of ±15 1 μg while that of androsterone (*sulfate*) *was 25 0 ± 10 7 μg* (*Migeon et al* 1957) These values are in good agreement with those reported by Kellie and Smith (1957) It must be noted that there were large individual variations There were also some day to day changes in the levels of each subject In addition it was observed that levels of peripheral plasma dehydroepiandrosterone (sulfate) followed a diurnal rhythm This variation was small but significant and could be the result of either (1) a change in rate of steroid production (2) a change in rate of steroid catabolism *by the liver or (3) a change in rate of steroid excretion* by the kidney Our present knowledge does not permit any conclusion (Migeon *et al* 1957)

As shown in Table III the concentrations for the 17 ketosteroids conjugated with glucuronic acid were low when compared with sulfates in the few cases reported

Levels of Peripheral Plasma 17 Ketosteroids in Normal Young Adult Females

The results obtained for the individual 17 ketosteroids of normal females were similar to those obtained in males The values for females were slightly *but not significantly lower than those for males* Table V indicates that the menstrual cycle had no significant effect on plasma

TABLE V Effect of the Menstrual Cycle on the Levels of Plasma Dehydro epiandrosterone (Sulfate) and Androsterone (Sulfate)

17 Keto teroid	2 Day afte beginning men	2 Week afte b ginni g men	3 W eks afte b g nning m ns	2 Day afte beginning f llowing men es
Dehydr epiandroste o e (ulfate?)	43 5 17 5† (16)‡	44 1 19 0 (15)	37 0 ± 16 9 (15)	33 2 16 4 (9)
Andro t ro e (ulfate?)	21 5 ± 12 3 (16)	21 7 8 2 (16)	17 0 8 3 (16)	18 9 13 1 (9)

Value w btained from 18 no mal f males 21 to 29 y ar f ag
†A ag l an ± tandard deviatio in ug pe 100 ml f plasm
‡Val indi te th mbe f d t minati as made in each group

dehydroepiandrosterone (sulfate) and androsterone (sulfate) contrary to what had been suggested from preliminary data

Effects of Aging

Figure 7 shows that the levels of dehydroepiandrosterone (sulfate) and androsterone (sulfate) were at their maximum between 20 and 39 years of age The fact that with the aging process plasma androgen levels decreased more rapidly than urinary androgens is probably due to the microtechnique for blood determination being less accurate and less sensitive than urine methods

In 12 postmenopausal women ranging from 46 to 95 years of age the peripheral plasma 17 ketosteroids were also very low (Migeon *et al* 1957)

Levels of Peripheral Plasma 17 Ketosteroids in Infants and Children

The values obtained for dehydroepiandrosterone (sulfate) and androsterone (sulfate) in infants and children are seen in Figure 8 The levels are compared with those found in umbilical cord plasma and in the plasma of normal adult males Following birth the concentrations fall rapidly to zero and stay at this level until the age of 5 to 7 years Subsequently the values rise progressively to adult levels (Migeon *et al* 1957) These results confirm those of Gardner and Walton (1954a) in reference to total plasma 17 ketosteroids in full term infants In addition the latter authors have reported that premature infants showed maximal values greater than the values shown

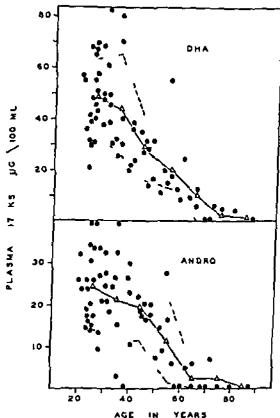


FIGURE 7 Plasma levels of dehydroepiandrosterone (sulfate) (DHA) and androsterone (sulfate) in normal males The black circles represent individual values The open triangles are the mean values at the various decades of age and the shaded area shows the ± values of a standard deviation from the mean From Migeon C J *et al* *J Clin Endocrinol and Metabolism* 17 1051 1957

by full term infants furthermore measurable concentrations still could be found up to 42 days of age contrasting with the prompt fall observed in full term infants

Effects of Pregnancy

Thirty pregnant women near term showed a mean plasma value of total 17 ketosteroids of 18 µg per hundred milliliters while adult controls had a mean value of 61 µg (Gardner *et al* 1954) Moreover in 19 paired specimens of blood from mother and cord it was found that the concentrations of neutral 17 ketosteroids in cord plasma were higher than those in maternal plasma (Gardner and Walton 1954b) Migeon *et al* (1955) have reported that dehydroepiandrosterone (sulfate) levels in cord plasma were similar to those found in normal

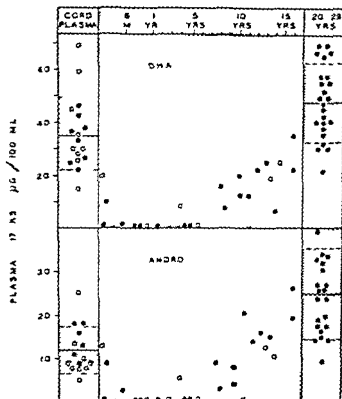


Figure 8 Plasma levels of dehydroepiandrosterone (sulfate) (DHA) and androsterone (sulfate) during childhood and adolescence. The open circles are the values obtained in females and the black circles are those obtained in males

adult individuals but higher than those of the respective mothers on the other hand androsterone (sulfate) levels were similar in maternal and cord plasma

It is impossible with the information at hand to explain the results reported above and to decide whether the fetal cortex produces the androgens found in the fetal circulation or whether these high concentrations are due to the accumulation of maternal androgens which crossed the placental barrier

Anencephaly

The 17 ketosteroid variations in the urine and plasma of the normal infant seem to parallel the involution of the fetal zone of the adrenal gland. Several androgens i e Δ^4 androstene 3 17-dione, 11β hydroxy

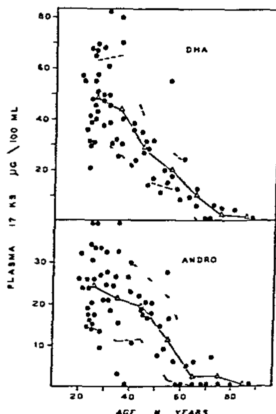


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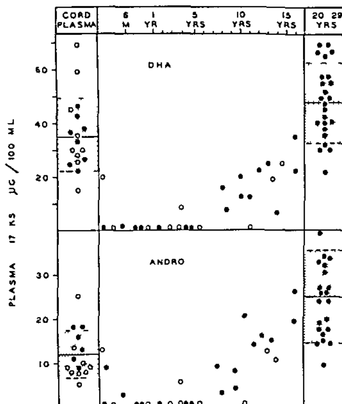


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Δ^4 androstene 3 17-dione and dehydroepiandrosterone have been detected in fetal adrenal glands (Bloch *et al* 1955 Bloch *et al* 1956) These findings tend to show that the fetal adrenal cortex is an active site of androgen production The anencephalic fetus has an almost complete absence of the fetal androgenic zone of the adrenal cortex In two such cases a decrease in plasma 17 ketosteroids was noted (Nichols *et al* 1958) further suggesting that the fetal zone is active in the synthesis of androgens However DiGeorge *et al* (1956) have reported the case of an anencephalic fetus which had high titers of 17 ketosteroids in plasma It is difficult to reconcile the findings of these two groups of investigators but it is evident that no definitive conclusion can presently be drawn from the study of a total of only 3 cases

Binding of Androgens to Human Plasma Proteins

Gardner (1954) has analyzed the various Cohn fractions for their 17 ketosteroid content Fractions IV 1 IV-4 and V showed the highest 17 ketosteroid concentrations On the other hand Sandberg *et al* (1951) reported variable results These same authors have observed that both steroids and their conjugates were bound to human plasma proteins particularly Fraction V the other fractions also being involved to various degrees in this process

Effect of ACTH

The intravenous infusion of 25 mg of ACTH over a period of 6 hours is known to produce an increase in plasma 17 hydroxycorticosteroids When administered in this manner ACTH increased also the levels of plasma dehydroepiandrosterone (sulfate) and androsterone (sulfate) Furthermore in some cases 11β hydroxy Δ^4 androstene 3 17 dione could be detected in the free fraction while androsterone and etiocholanolone were found in the glucuronoside fraction none of these steroids had been visualized prior to ACTH administration in extracts of similar volume of plasma However the amplitude and time of maximum response were not as regular as those for 17 hydroxycorticosteroids An increase in the dose of ACTH administered did not modify the results obtained Moreover some normal subjects did not demonstrate any significant response (Migeon 1955) Ceresa and Cravetto (1955) have found a variable response of dehydroepiandrosterone and androsterone levels following administration of ACTH

Effect of Human Chorionic Gonadotropin

An increase in the concentration of plasma androsterone has been reported by Eik Nes *et al* (1959) following the administration of chorionic gonadotropin to normal human males This increase was not observed in 5 normal young females The method of measurement of plasma 17 ketosteroids used in this study was that of Oertel and Eik Nes (1958)

Plasma 17 Ketosteroids in Anuria

The patient studied by Savard (1957) was a 22 year-old woman with acute renal tubular necrosis due to a massive hemorrhage following an abruption of the placenta The patient was almost anuric with extreme uremia and was placed on the artificial kidney The 100 liters of rinsing fluid were analyzed for their 17 ketosteroid content Of a total of 7 41 mg of 17 ketosteroids 5 5 mg were unconjugated 1 6 mg were glucuronosides and 0 31 mg non glucuronosides The free and glucuronoside fractions yielded 11β hydroxy androsterone 11β hydroxy-etiocholanolone and either 11 keto-etiocholanolone or 11 keto androsterone The non glucuronoside fraction contained androsterone and small amounts of other compounds

These results probably reflect detention in the circulation of steroids which are normally rapidly cleared by the kidney In addition the patient had just terminated a pregnancy a condition which is associated with a decrease of the dehydroepiandrosterone levels in plasma

Syndrome of Virilism

Several androgens have been detected in ovarian vein blood from patients with virilizing ovarian tumors (Table II) However the study of peripheral plasma did not demonstrate any notable increase of the 17 ketosteroid glucuronosides It must be added that the increase in urinary 17 ketosteroids in such cases is often minimal

In congenital adrenal hyperplasia there was some increase of the androgen sulfates (Migeon 1955) androsterone etiocholanolone and a certain amount of 11-oxy 17 ketosteroids were also found in the glucuronoside fraction Concentrations of total plasma 17 ketosteroids in this condition were found to vary between 80 and 360 μg per hundred milliliters (Gardner 1953) Cortisone therapy brought a prompt fall of the levels similar to that observed for the urinary 17 ketosteroids as shown on Figure 9 (Gonzales and Gardner 1956)

Δ^4 androstene 3 17 dione and dehydroepiandrosterone have been detected in fetal adrenal glands (Bloch *et al*, 1955 Bloch *et al* 1956) These findings tend to show that the fetal adrenal cortex is an active site of androgen production The anencephalic fetus has an almost complete absence of the fetal androgenic zone of the adrenal cortex In two such cases a decrease in plasma 17 ketosteroids was noted (Nichols *et al* 1958) further suggesting that the fetal zone is active in the synthesis of androgens However DiGeorge *et al* (1956) have reported the case of an anencephalic fetus which had high titers of 17 ketosteroids in plasma It is difficult to reconcile the findings of these two groups of investigators but it is evident that no definitive conclusion can presently be drawn from the study of a total of only 3 cases

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jugates is responsible for the preponderance of sulfates in peripheral circulation Also observations on the physiological and pathological variations of plasma androgens have been reported

Despite all these advances there are still many problems to be solved

It must be stressed again that the active hormones are the noncon jugated steroids while most of the androgens measured in blood are present as conjugates Consequently our present methods do not determine the degree of androgenicity available at the target tissue

Urinary 17 ketosteroids measure only a small fraction of the metabolites excreted by the kidney and it is doubtful that in all cases one can relate this fraction to the total production of the active precursor Conjugated blood androgens are even more confusing since some groups of conjugates are cleared from the circulation more rapidly than other groups

Finally it is difficult to imagine that the difficult techniques of measurement of plasma androgens can be simplified On the contrary in order to obtain better information from such studies it will be necessary to analyze separately the free glucuronoside and sulfate fractions and to determine the qualitative as well as quantitative con tent of each of these fractions Efforts will have to be made to devise microtechniques for the identification of the individual steroids

Because of these difficulties it is almost certain that the determina tion of plasma androgens will not become a routine laboratory procedure Nevertheless as a research tool it will further increase our knowledge of the physiological and pathological metabolism of andro gens in man

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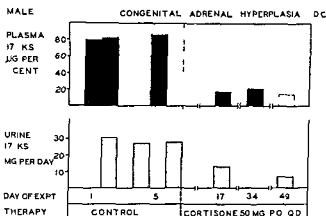


FIGURE 9 Effect of cortisone therapy on plasma and urinary 17 ketosteroids in a 5 year old boy with congenital adrenal hyperplasia (From Gonzales R F and Gardner L I *Pediatrics* 17 524 1956)

In Cushing s syndrome 11β hydroxy Δ^4 androstene 3 17 dione was detected in the free extract of most patients in addition etiocholanolone was also found in one patient who was receiving ACTH The glucuronoside fraction in contrast to the results found in normal subjects often contained more 17 ketosteroids than the sulfate fraction with proportionally large amounts of 11 hydroxy androsterone 11 keto etiocholanolone and etiocholanolone and smaller quantities of 11 hydroxyetiocholanolone androsterone and a compound less polar than androsterone (Migeon and Lescure)

CONCLUSIONS

Prior to 1953 very little was known about plasma androgens in man except for the demonstration of biological androgenic activity in blood extracts Since then a large body of information has been made available Androgens have been detected or isolated from adrenal ovarian and spermatic vein blood Incubation or perfusion studies have suggested that the biosynthesis of androgens in different endocrine glands might follow a similar pathway progesterone being an important precursor Dehydroepiandrosterone and androsterone have been isolated from peripheral human plasma after continuous ether extraction at pH 0 8 while small amounts of C_{19} steroids have been found following β glucuronidase hydrolysis and minute amounts of free androgens have been detected Metabolic studies have demonstrated that the difference in renal clearance of sulfate and glucuronoside con

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CHAPTER XI

Corticosteroids in Blood*

Don H. Nelson, Kristen Eik-Nes *and* Avery A. Sandberg

SECTION 1 HORMONAL SECRETION OF THE ADRENAL CORTEX

Don H. Nelson

- Hormones isolated from adrenal venous blood
- Substances influencing secretion of hormones into the adrenal venous effluent
- Levels of corticosteroids in blood
  - Normal levels
  - Diurnal variation
  - Age
  - Pregnancy
  - Changes in adrenal secretion and peripheral blood levels with stress
- Evaluation of normal adrenal secretion
  - ACTH test
  - Suppression of adrenocortical secretion by the administration of corticosteroids
  - Sodium deprivation test for aldosterone secretion
- Pathological disorders of adrenal secretion
  - Addison's disease
  - Cushing's syndrome
  - Primary hyperaldosteronism
  - Primary hypoaldosteronism
  - Secondary hyperaldosteronism
  - Adrenogenital syndrome

SECTION 2 METHODS FOR MEASUREMENT OF CORTICOSTEROIDS IN BLOOD

Kristen Eik-Nes

- Introduction
- Extraction of adrenal steroids from human blood plasma
- Purification of adrenal steroids in extracts of human blood plasma
- Identification and quantitative determination of adrenal steroids in purified extracts of human blood plasma

* The large number of publications in this field during the past decade precludes the inclusion of all the references to this subject. The interested reader is referred to other reviews of this subject for further details (Nelson, 1956a, b; Borth, 1957; Gold, 1957).

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CHAPTER XI

Corticosteroids in Blood*

Don H Nelson, Kristen Eik Nes and Avery A Sandberg

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Don H Nelson
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Substances influencing secretion of hormones into the adrenal venous effluent
Levels of corticosteroids in blood
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Purification of adrenal steroids in extracts of human blood plasma
Identification and quantitative determination of adrenal steroids in purified extracts of human blood plasma

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Section 3 THE INFLUENCE OF EXTRA-ADRENAL FACTORS ON BLOOD CORTICOSTEROIDS

Avery A Sandberg

The rate of metabolism and the miscible pool of cortisol
- Diurnal variation
- Effects of age
- Effects of liver function
- Effects of thyroid function
- Effects of renal disease
- Pregnancy
- Effects of stress
- Effects of medication and adrenal insufficiency

Corticosterone

Aldosterone

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Section 1

Hormonal Secretion of the Adrenal Cortex

Don H Nelson

HORMONES ISOLATED FROM ADRENAL VENOUS BLOOD

If the assumption is made that those compounds appearing in adrenal venous blood in higher concentration than in the peripheral circulation are those substances produced and secreted by the adrenal cortex an examination of such adrenal effluent should reveal the natural secretion of the adrenal cortex Such studies have been carried out in a wide variety of species and although species differences in relative proportions of the various steroids do exist it appears that the same primary compounds are secreted by all of the species thus far examined These may be divided conveniently into three primary groups (1) those present in largest quantity are represented in the human by the secretion of large quantities of hydrocortisone and corticosterone and are the 11 hydroxy 18 desoxy compounds (2) there is the secretion of aldosterone which contains an oxygen in the 18 position in contrast to the aforementioned compounds and (3) there are a group of C_{19} compounds which are commonly referred to as the adrenal androgens (see Fig 1)

It is established that 17 hydroxycorticosterone (11β 17α 21 trihydroxy 4 pregnene 3 20-dione hydrocortisone cortisol Compound F) is a primary secretory product of the human adrenal cortex (Nelson and Samuels 1952 Bush and Sandberg 1953 Romanoff *et al* 1953 Hudson and Lombardo 1955) The best estimates of total secretion of this compound indicate that approximately 25 mg of hydrocortisone is secreted by the adrenal cortex during an average 24 hour period (Bondy and Altrock 1953) This substance represents the chief gluco corticoid produced by the adrenal cortex and is undoubtedly chiefly responsible for the metabolic effects of this type produced by the adrenal gland Estimations of the quantity of corticosterone (11β 21 dihydroxy 4 pregnene 3 20 dione) produced per day are not as clearly in agreement as are those for hydrocortisone but it is estimated that the quantity produced is in the neighborhood of 2 to 3 mg per 24 hours (Peterson 1957a) This makes the ratio of secretion of hydro cortisone to corticosterone approximately 10 1 In addition to these two compounds which are produced in the largest quantity various

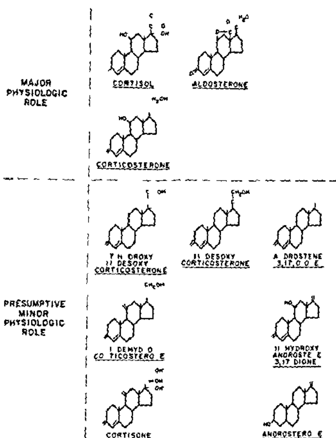


FIGURE 1 Secretory products of the human adrenal cortex

workers have reported the presence of much smaller amounts of 17 hydroxy 11 desoxycorticosterone (17α 21 dihydroxy 4 pregnene 3 20 dione Compound S) 11 dehydrocorticosterone (21 hydroxy 4 pregnene 3 11 20 trione Compound A) 17 hydroxy 11 dehydrocorticosterone (17α 21-dihydroxy-4 pregnene 3 11 20 trione Compound E or cortisone) and 11 desoxycorticosterone (17α 21-dihydroxy 4 pregnene 3 20 dione DOC) Compound S was found in a total dose of 1 3 μg per milliliter of adrenal venous blood of humans by Touchstone (1958) and both Compounds E and A have been reported in the peripheral blood of man (Morris and Williams 1953a) and have been assumed to be minor secretory products of the adrenal cortex Desoxycorticosterone has not been reported in the adrenal venous or peripheral blood of humans Only relatively small amounts of this effluent blood have been available for study and very small quantities of this

steroid have been found in large amounts of such blood obtained from the adrenal vein of other species (Bush 1951 Farrell and Lamus 1953) It appears likely that such compounds may be secreted in very small but probably not physiologically significant quantities by the human adrenal cortex Since aldosterone (11β 21 dihydroxy-4 pregnene 18 al 3 20-dione) was first found in adrenal venous blood (*Simpson et al* 1952) it has become evident that this compound is the chief mineralocorticoid secreted by the adrenal cortex not only in the human but in many other species as well This compound differs from the other known steroids produced in the human by the presence of the C_{18} aldehyde group and also by the extreme potency of this substance in comparison with the other corticosteroids in producing physiological effects on renal handling of sodium and potassium The best accepted estimations are that approximately 200 μg of this compound are secreted per 24 hours by the normal unstressed human being (Ayres *et al* 1957) Although aldosterone administered in quantities comparable to that of hydrocortisone has similar potency in assays of adrenal activity such as glycogen deposition the extremely small quantity of this substance produced by the adrenal cortex in man makes it unlikely that aldosterone exerts any significant physiological effect on glucocorticoid parameters

Although there has been considerable difference of opinion in the past regarding the question of adrenal androgen production there is now little doubt that the adrenal cortex of man is able to produce steroids having significant androgenic effects Androgens are produced by the adrenal gland physiologically as well as in certain pathological conditions (see p 347) Four substances of this general type have been isolated from human adrenal venous blood These are 4 androstene 3 17 dione 11β hydroxy 4 androstene 3 17 dione androsterone (3α-hydroxy androstene 17 one) and possibly dehydroisoandrosterone (3β hydroxy 5 androstene 17 one) (Romanoff *et al* 1953 Pincus and Romanoff, 1955 Bush *et al* 1956) Although androsterone is the most potent of these androgens each may have some biological androgenic activity and contribute to the total androgenicity of the adrenal cortical secretion The entire subject of androgens in blood is discussed more fully by Migeon (Chapter X)

Of interest is a recent abstract which reports increased levels of estrogens in adrenal venous blood as compared with peripheral blood (Hardy and Ward 1958) The suggestion of estrogen secretion from adrenal tissue is in agreement with the findings of West *et al*

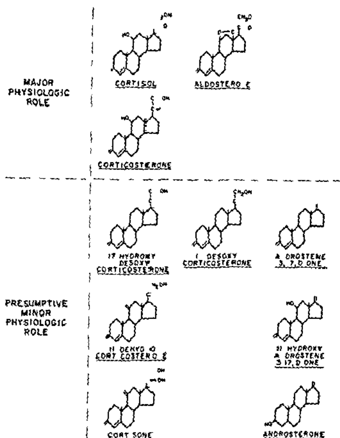


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human peripheral blood have been noted 15 to 20 minutes following the administration of ACTH or a pituitary stimulating stress (Nelson *et al* 1951) and thus would suggest that equally rapid secretion of corticosteroids may occur from the adrenal gland of the human being As little as 0 1 unit of ACTH given in a single injection into the general circulation of normal young subjects produced a reproducible increase in corticosteroids in the peripheral blood (Bliss *et al* 1954a) and 2 5 units given in a continuous infusion caused a significant increase in corticosteroid production over a 24 hour period (Nelson *et al* 1951)

Secretion of androgens by the adrenal cortex is also under the control of the pituitary gland Administration of ACTH produces an increase in secretion of androgens in the adrenal venous blood and removal of the pituitary gland causes a marked fall in production of such substances Not fully settled however is the suggestion that gonadrotrophins as well as ACTH produced by the anterior pituitary gland may influence the secretion of these androgenic hormones No increase was noted following the infusion of a chorionic gonadotrophin preparation into a single patient by Kappas *et al* (1956) Better evidence for an increased secretion of androgens has been noted following the administration of ACTH to normal subjects (Harrison *et al* 1954)

Those factors which control the secretion of aldosterone are even less clearly delineated Although studies have demonstrated stress factors which influence the production of aldosterone as yet a specific chemical compound produced by a higher center which may control the secretion of aldosterone has not been identified It is true however that administration of ACTH will under certain circumstances influence such production of aldosterone (Muller *et al* 1956) It is also clearly demonstrated that no substance produced by the anterior lobe *of the pituitary gland is necessary for the control of aldosterone production* Hypophysectomized subjects clearly respond to the various stresses which control aldosterone production (Luetscher and Axelrod 1954) Although *in vitro* studies have suggested that sodium/potassium ratios in the fluid perfusing the adrenal cortex may also influence such secretion (Rosenfeld *et al* 1956) studies *in vivo* have not thus far substantiated this ratio as a controlling factor of adrenal aldosterone production

(1958) who described increased estrogen excretion following ACTH administration to a patient with adrenocortical carcinoma and Cushing's syndrome

SUBSTANCES INFLUENCING SECRETION OF HORMONES INTO THE ADRENAL VENOUS EFFLUENT

Control of the secretion of hydrocortisone corticosterone and the 11 hydroxy 18-desoxysteroids produced by the adrenal cortex is undoubtedly almost completely through the pituitary gland as mediated by the secretion of adrenocorticotrophin (ACTH) Many studies on small animals have demonstrated the almost complete dependence of the adrenal cortex on the secretion of ACTH by the anterior lobe of the pituitary gland (Sayers 1950) It is of interest however that the hypophysectomized human being or small animal gets along much better than does the adrenalectomized subject and indeed can be maintained without hormonal replacement This may be due largely to the fact that aldosterone secretion is not chiefly controlled by a substance secreted by the pituitary gland Although hypophysectomized or panhypopituitary subjects have very low levels of corticosteroids in their blood or urine such substances are usually not completely absent (Sweat and Farrell 1954) Small quantities of these compounds may be produced by the adrenal cortex in the absence of direct control by ACTH secreted by the anterior lobe of the pituitary gland but *little or no* increase in secretion may be obtained following stress The lack of sensitivity of the adrenal cortex of the hypophysectomized individual to the administration of ACTH is discussed further under Evaluation of Normal Adrenal Secretion (see p 344)

Due to the obvious complications of attempting to carry out physiological studies on human adrenal venous blood few such investigations are available Studies in the dog and other species however have demonstrated the extreme sensitivity of the adrenal cortex to ACTH the immediate response which may be obtained following the administration of ACTH *and the relatively short period of action* of this substance when given in very small doses Thus 1 milliunit or less of ACTH administered into the general circulation of a 20 Kg dog produces an increase in secretion from the adrenal cortex within 3 minutes and this added secretory activity diminishes to control levels within 30 minutes following the administration of the compound (Nelson and Hume 1955) Increases of corticosteroids in

to a number of physiological factors which apparently influence the secretion of these compounds These include diurnal variation age pregnancy and a large number of stresses which undoubtedly influence secretion from the adrenal cortex and therefore also produce changes in the concentration of the compound in blood or plasma

Diurnal Variation

Studies of urinary and plasma levels of corticosteroids taken at intervals through an entire 24 hour period have repeatedly demonstrated that there is a diurnal variation in the secretion of these substances Thus it was noted that blood levels obtained at 8 a m were higher than those found later in the day (Bliss *et al* 1953) These studies were later extended to include samples taken at intervals of a few hours throughout the day and night Such studies demonstrated that blood levels were highest in the early morning the peak usually being found at about 6 a m these levels then decreased gradually through the day hitting a low point about 10 to 12 p m after which they again began to rise to the 6 a m peak Although the validity of such a diurnal variation is unquestioned the factor or factors which control such variation are less clear Migeon *et al* (1956a) undertook a study of secretion of these substances in night workers and blind subjects in order to determine whether light and activity may have an influence on the diurnal variation Normal subjects who had been working at night for at least six months were subjected to the estimation of blood corticoids in a similar manner as were completely blind individuals In neither case was any variation in the normal diurnal pattern found Although such studies have not been carried out in man with the estimation of steroids secreted in adrenal venous blood the close similarity between urinary excretion and blood levels of these compounds makes it appear likely that the changes seen are due to production rather than to any change in metabolism of the corticoids which may occur during the diurnal period

Age

Little difference has been noted between peripheral blood levels of 17 hydroxycorticosteroids in children and in young or elderly adults Blood levels in newborn subjects however have been reported to be as low as 0 9 μg per hundred cubic centimeters of plasma (Klein *et al* 1954) and urinary excretion of corticosteroids has been noted to be somewhat lower in elderly subjects than in young adults Although

LEVELS OF CORTICOSTEROIDS IN BLOOD

Normal Levels

Secretion by the adrenal cortex of extremely small quantities of steroids plus the above described multiplicity of these compounds makes extremely difficult an adequate determination of the exact physiological levels of the corticosteroids in peripheral blood of man. Table I lists the values which have been found by a number of workers

TABLE I. 17-Hydroxycorticoids in Peripheral Blood of Patients with Addison's Disease and Cushing's Syndrome (μg/100 ml plasma)

Diagnosis	Control Mean or Range	After ACTH Mean or Range	Investigators
Cushing's syndrome (hyperplasia)	28 (16–34)	81 (61–118)	Christy et al. (1957)
Cushing's syndrome (adenoma or carcinoma)	42 (28–68)	51 (34–73)	
Normal	16	46 (35–55)	
Cushing's syndrome	35–62	130–140	Sandberg et al. (1957a)
Normal	11	40	
Addison's disease	0.4–4.0	0–5.2	
Addison's disease	0–14	0–14	Eik-Nes et al. (1955a)
Normal	3–22	25–50	
Addison's disease	0–17	–	Perkoff et al. (1954)
Cushing's syndrome	27–103	–	
Normal	13	–	
Normal	8–20	–	Nelson et al. (1951)
Addison's disease	0	–	

for those corticosteroids which are found in largest quantity and therefore may be measured in peripheral blood. It will be noted that those compounds which are most commonly measured are the so called 17-hydroxycorticosteroids as determined by the colorimetric reaction of Porter and Silber (1950) (see Section 2). The rather wide range of values found by various workers is due not only to changes which may occur in the chemical estimations of these compounds but also

to a number of physiological factors which apparently influence the secretion of these compounds These include diurnal variation age pregnancy and a large number of stresses which undoubtedly influence secretion from the adrenal cortex and therefore also produce changes in the concentration of the compound in blood or plasma

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initial levels in young subjects have been low a normal response to the administration of ACTH or the stress of operation has been seen (Klein and Rovanek 1956) Similarly although blood levels of 17 hydroxycorticosteroids are within the normal range for young adults the rate of metabolism of these substances has been shown to be markedly different in the elderly group Studies carried out by Samuels (1957) suggest that there is a smaller volume of distribution for these substances in the elderly group and therefore although less corticoid is produced approximately the same levels are maintained in peripheral body fluids (See Section 3 for a more complete explanation of changes seen)

Pregnancy

A progressive increase in plasma corticoid levels during pregnancy has been noted by a number of workers Gemzell (1953) found normal mean levels to be 5 to 6 μg per hundred milliliters of plasma drawn at 9 30 p m in nonpregnant subjects and found a progressive increase during pregnancy from these levels to levels of 10 to 35 μg near term Similarly Robinson *et al* (1955) who found mean normal blood levels to be 12 μg per hundred milliliters of plasma noted an increase to a mean of 33 μg during pregnancy with the highest levels being found during the latter months Little *et al* (1958) also found higher levels of 17 hydroxycorticosteroids present during the third than the first or second trimesters of pregnancy Failure to find consistent elevation in total urinary corticosteroids (Norymberski *et al* 1953) led Migeon *et al* (1957) to investigate the physiological disposition of cortisol 4 C^{14} during late pregnancy The demonstration of a half life of free radioactive tagged hydrocortisone which was twice that observed in control subjects suggests that this factor of metabolism may explain to a large extent the increases in plasma corticoid seen in pregnancy rather than an actual increase in production of these compounds by the adrenal cortex

Change in Adrenal Secretion and Peripheral Blood Levels with Stress

Although the term stress has become intimately associated with the adrenal cortex and stimulation of its secretion (Selye 1950) no completely satisfactory explanation of the mechanism by which stress produces an increase in corticosteroid secretion has been given There is little doubt that most of these stresses act by means of stimulating

the pituitary gland to secrete ACTH but the relationship of such ACTH stimulation to circulating levels of blood corticoids is far from clear Sayers (1950) suggested that a feedback mechanism worked on the anterior lobe to control such secretion The possibility was put forth that stress caused increased utilization of corticosteroids a resultant fall in blood corticoids occurred and that this decrease in corticoids then acted on the pituitary gland to produce an increase in ACTH production Measurement of simultaneous blood levels of corticosteroids and ACTH have demonstrated that in conditions of severe stress both may be markedly elevated and thus one would have to postulate other mechanisms for the stimulation of ACTH production under these circumstances (Hume and Nelson 1955) Similarly when blood levels of corticosteroids have been measured following various stresses there has been no evidence of an initial fall in blood corticoid levels prior to the commonly seen increase Although epinephrine has been suggested as a stimulator of the adrenal cortex (McDermott *et al* 1950) there is little evidence at present that this substance plays a physiologically significant role in the stimulation of human ACTH and adrenal secretion Following the administration of epinephrine to normal subjects in small to large doses no increase in blood or urinary corticosteroids was seen (Sandberg *et al* 1953b) Of the many stresses which are known to act as a stimulus to increased secretion of corticosteroids and thereby increase the circulating level of these hormones major surgery and severe tissue trauma are perhaps the most consistent in producing these effects (Franksson and Gemzell 1953 Sandberg *et al* 1954a Moore 1957) Some of the highest and most prolonged elevations of such hormones in blood have been seen following severe burns (Hume *et al* 1956) It is of interest that aldosterone as well as 17 hydroxycorticoids and 17 ketosteroids are usually elevated immediately following major surgery but aldosterone has been reported to remain at an increased level for a shorter period of time (Venning *et al* 1958) In these studies normal sodium retention did not occur regularly with the increase in aldosterone level and in fact was noted to occur at a later time when aldosterone levels had returned to normal These findings are contrary to those of Llaurado and Woodruff (1957) who correlated the post operative sodium retention with an increase in aldosterone secretion

A large number of physiological stresses have been reported to produce an increase in plasma corticoids Among these may be men tioned hypoxia and heat (Hale *et al* 1957) hypoglycemia and mus

cular exercise (Froesch *et al* 1954) It is of interest that these stresses generally failed to produce an increase in plasma corticoids following short or moderate exposure but produced such changes when the severity of the change from normal was progressively increased These changes would seem to reflect the protective aspects of adrenal cortical secretion on maintenance of homeostasis

In addition to these physiological stresses a number of chemical substances have been shown to produce an increase in levels of 17 hydroxycorticosteroids in the peripheral blood of man Among these may be listed insulin histamine bacterial pyrogens Antabuse alcohol reaction and such stresses as radiation with x ray and electroshock (Bliss *et al* 1954b Bliss *et al* 1954c French *et al* 1955) Of some interest is the fairly marked increase in plasma levels of corticosteroids which is noted commonly in terminally ill patients (Nelson *et al* 1951) This increase is due in part to changes in metabolism as is discussed further below (Section 3) but may also represent some increase in corticosteroid secretion associated with the anoxia or other physiological disturbances which are often present in such patients

Those stresses which stimulate the production of aldosterone do so by mechanisms separate from ACTH although in some cases some slight increase may be produced by ACTH discharge Almost any stress which causes a significant degree of fluid loss from the body has been shown to be a potent stimulus to aldosterone production Thus salt restriction hemorrhage and exposure to increased temperature with concomitant loss of fluid and electrolytes all may result in an increase in aldosterone secretion chiefly as noted by increase in urinary secretion but presumably an increase in blood aldosterone also occurs (Bartter *et al* 1956)

EVALUATION OF NORMAL ADRENAL SECRETION

A number of physiological tests of adrenal function have been employed clinically in order to determine the secretory capacity or the control of secretion of the adrenal cortex in certain physiological and pathological conditions Although these often have been standardized according to urinary levels rather than blood levels of corticoids in both instances an attempt is made to evaluate the secretion of the cortex under particular conditions and therefore such tests deserve some attention in our discussion of the secretion of the adrenal cortex

ACTH Test

There have been many descriptions of the use of ACTH administration in the evaluation of adrenal function The most widely employed of these consists of the intravenous administration of 25 units of standard ACTH over an 8 hour period with collection of a 24 hour urine sample (Laidlaw *et al* 1955 Thorn *et al* 1956) This test is carried out on at least two and in some cases three successive days and the increase of urinary 17 hydroxycorticoids and 17 ketosteroids over baseline levels is used as an indication of the functional capacity of the adrenal cortex Similar studies have been carried out in which ACTH is given intravenously in large or small doses and the concentration in peripheral blood of various corticosteriods chiefly 17 hydroxycorticoids is followed over a period of minutes to hours A rough approximation of the ability of the adrenal cortex to secrete 17 hydroxycorticoids may be obtained in this way and indeed it may even be used as an assay of ACTH (Bliss *et al* 1954a) The lambda values of such an assay are rather poor however and other methods give more precise results An assay using the corticoid secretion into the adrenal venous blood of hypophysectomized dogs does serve as a valid assay of ACTH (Nelson and Hume 1955)

A comment concerning the relative validity of blood and urinary corticoids in such tests should perhaps be made The excretion of corticoids over an entire 24 hour period seems to be a better estimation of total secretion over such periods than would be isolated blood levels It has been noted however that small increases in corticoids which may occur following minimal stresses or the administration of very small quantities of ACTH result in an elevation of blood corticoids at a time when variations in urinary corticoids would be too small to be of statistical significance It is important therefore in undertaking the estimation of adrenal function by tests such as these that one have clearly in mind the dynamic aspects of this system and the rapidity with which blood levels of these compounds may change

Suppression of Adrenocortical Secretion by the Administration of Corticosteroids

Another means of estimating those factors which are controlling secretion of the adrenal cortex is the administration of some bio

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PATHOLOGICAL DISORDERS OF ADRENAL SECRETION

Addison s Disease

Adrenal insufficiency may be associated with little or no secretion of adrenal cortical steroids due to idiopathic atrophy of the adrenal gland or destruction of the gland by some infectious or invasive process It is rare however to find a patient who secretes no corticosteroids under these circumstances It is usually possible to measure some minimal secretion of these hormones The observation that some of these patients may have normal secretion of corticosteroids per 24 hours with essentially normal levels of these hormones in blood and urine led to the designation of partial Addison s disease to describe some of these cases (Perkoff *et al* 1954 Abu Haydar *et al* 1958) Such patients were found to be secreting at maximal capacity and further administration of ACTH failed to cause further increase in corticosteroid production These findings suggested that the pituitary gland was probably putting out excess quantities of ACTH which were stimulating the adrenal cortex maximally and therefore further ACTH would fail to cause an increase in cortical secretion This concept has recently been verified by the demonstration of increased levels of ACTH circulating in the peripheral plasma of such patients (Bethune *et al* 1957) There is no evidence that there are any qualitative alterations in adrenal cortical secretion in cases of either complete or partial adrenal insufficiency

Cushing s Syndrome

Cushing s syndrome due to adrenal hyperactivity may have as its etiological basis and as its predominant presenting symptom many different factors Those aspects of adrenal hyperactivity associated chiefly with excess androgen or aldosterone production are considered separately so this section is devoted chiefly to increases in compounds of the cortisol or cortisone type Adrenal hyperactivity of this type may be produced by increased production of ACTH such as by a tumor of the anterior lobe of the pituitary gland or other factors stimulating ACTH production by a benign or malignant tumor of the adrenal cortex or in rare cases by aberrant tissue of adrenal cortical type which may be found in ovarian or other areas In the classic syndrome markedly increased secretion of corticosteroids results in elevated levels of corticosteroids in both blood and urine Patients with malignant

logically potent corticoid with estimation of blood and urinary corticosteroids during this period of administration The substances administered are those such as 9α fluorohydrocortisone which contribute little to blood or urinary levels of corticosteroids in the dosage given but are excellent inhibitors of the production of ACTH by the pituitary gland when given in quantities of only a few milligrams per day Thus 5 to 10 mg of such a substance is given daily usually in two divided doses over a period of 3 to 5 days *A decrease in secretion* of the adrenal cortex is interpreted to mean that ACTH secretion has been suppressed and that the corticoids being produced are controlled by such ACTH production (Laidlaw *et al* 1955 Thorn *et al*, 1956) Failure to obtain suppression is usually thought to be indicative of autogenous production of hormones by the adrenal cortex such as might be seen with a benign or malignant tumor of this organ

Sodium Deprivation Test for Aldosterone Secretion

The secretion of aldosterone by the adrenal cortex as noted above is not chiefly under the control of the anterior lobe of the pituitary gland or of ACTH and thus the above mentioned tests are of little value in determining the functional ability of the adrenal cortex *to secrete aldosterone As has been noted however one of the most* potent stimulators of aldosterone production is the removal of sodium from the diet A number of investigators therefore have used a test which consists of a few days during which aldosterone is estimated in the urine and sodium intake is fairly high followed by a few days during which the sodium intake is markedly reduced and aldosterone is again determined in the urine as a physiological means of testing the ability of the adrenal to increase aldosterone secretion *Such a* test is obviously contraindicated in a subject who cannot maintain sodium balance on normal sodium intake and might be harmful in a patient with Addison s disease or a sodium losing renal lesion With such a test however aldosterone should increase in the urine and has been used as a means of helping to delineate the parameters of a syndrome which has been called primary hypoaldosteronism (Hudson *et al* 1957) Normal individuals increase aldosterone production by a factor of two to four within 3 days of being placed on a sodium intake of approximately 10 mEq per day (Hernando *et al* 1957)

also been seen In such cases increases of aldosterone production of the nature of two to fourfold have been noted and this excess production has disappeared following removal of the tumor (Conn 1955 Bartter and Biglieri 1958)

Primary Hypoaldosteronism

The possibility that there might be a specific defect in aldosterone production in a particular patient was investigated by Hudson *et al* (1957) These authors have described a syndrome apparently due to lack of production of aldosterone in response to the stress of sodium deprivation If such a defect occurs in adrenal secretion it appears to be very rare such a diagnosis should be made only after careful evaluation of aldosterone and total steroid secretion In the case described normal quantities of 17 hydroxycorticoids and 17 ketosteroids were produced and these increased normally following stimulation by ACTH

Secondary Hyperaldosteronism

The presence of a number of conditions in which aldosterone may be secreted in excess as shown by isotope turnover studies and excess quantities of aldosterone in the urine has been well described The conditions most commonly referred to are those of congestive heart failure cirrhosis with ascites and nephrosis although other conditions such as idiopathic edema have been included At present there is no evidence that these conditions are associated with any pathological changes in the adrenal cortex and the hyperaldosteronism appears to be due to indirect stimulus or hormonal secretion by a factor or factors which are as yet poorly understood

Adrenogenital Syndrome

Qualitative as well as quantitative changes in secretion of the adrenal cortex have been noted in those patients having the adrenogenital syndrome Lewis and Wilkins (1949) and others noted that when ACTH was administered to patients with congenital adrenal hyperplasia there was a considerable increase in the already elevated urinary 17 ketosteroids and there was not a normal increase in other urinary corticosteroids Failure to observe an increase in blood corticoids following the administration of ACTH to such patients again suggested the possibility of some qualitative derangement of corticoid secretion (Kelley *et al* 1952) However the administration of corti

tumors are likely to show the highest values, and thus values in the neighborhood of 100 μg or more of 17 hydroxycorticosteroids per milliliter of blood have been noted in such conditions Such tumors are usually not responsive to the administration of ACTH and will not suppress when the suppression test is carried out Qualitative as well as *quantitative* changes in secretion may be seen thus it is not uncommon to have a preponderance of either 17 hydroxycorticoids of the hydrocortisone type or of androgens of the androsterone variety (Laidlaw *et al* 1955) It has also been noted that these patients may secrete increased quantities of compounds such as Compound S and dehydroisoandrosterone (Dohan *et al* 1955) Patients with single benign adenomas of the adrenal cortex may secrete increased quantities of either hydrocortisone or androgens but such patients presenting the clinical picture of Cushing s syndrome are more likely to secrete a preponderance of hydrocortisone Indeed the secretion of larger quantities of this compound than of the androgens may be used as suggestive evidence of an adenoma of the adrenal cortex being present A majority of patients with Cushing s syndrome have only adrenal *hyperplasia which is bilateral in nature and pathologically indistinctive* except for the usual marked increase in weight of the adrenal glands Such patients respond hyperactively to the administration of ACTH and secretion of corticoids can usually be suppressed with the suppression test Unfortunately such rules do not always apply and patients with adrenal hyperplasia and Cushing s syndrome whose corticoid secretion cannot be suppressed or who are not hyperactively responsive to ACTH administration have been described (Jenkins and Spence 1957) Nevertheless the failure to respond to ACTH and lack of ability to suppress are suggestive evidence in favor of *adenoma the converse being usually found in* patients with adrenal hyperplasia Blood levels of adrenal steroids (17 hydroxycorticoids are usually measured) are high normal to elevated in this condition and it has been noted that the normal diurnal variation in corticoid secretion may be reduced or absent (see Table I)

Primary Hyperaldosteronism

Although the secretion of increased quantities of aldosterone may occur in Cushing s syndrome of the type described above a primary increase in aldosterone produces a well described clinical syndrome Such hyperaldosteronism has generally been found to be due to an adrenal adenoma but a few cases due to adrenal hyperplasia have

Section 2

Methods for Measurement of Corticosteroids in Blood

Kristen Eik Nes

INTRODUCTION

Cortisol (11β 17α 21 trihydroxy-4 pregnene 3 20 dione) and corticosterone (11β 21-dihydroxy-4 pregnene 3 20 dione) have been isolated and identified in adrenal venous effluent from human subjects with malignant diseases (Pincus and Romanoff 1955) The data indicate that cortisol corticosterone and aldosterone (11β 21 dihydroxy-4 pregnene 18 al 3 20 dione) are present in the systemic circulation of normal man in such a form that they can be extracted from plasma without hydrolysis (Nelson and Samuels 1952 Bush and Sandberg 1953 Simpson and Tait 1955 Sweat 1955 Petersen 1957a) By the criteria of paper chromatography and staining characteristics Eberlein and Bongiovanni (1956) have indicated that Compound S (17α 21 dihydroxy-4 pregnene 3 20-dione) was present in the peripheral blood of an 8 year-old pseudohermaphrodite girl with congenital adrenal hyperplasia and hypertension Several investigators have presented evidence for adrenal steroid hormones other than cortisol corticosterone and aldosterone as begin present in either human adrenal vein blood or human peripheral blood (Bush and Sandberg 1953 Hudson and Lombardo 1955 Pincus and Romanoff 1955 Sweat 1955 Weichselbaum and Margraf 1957 Touchstone 1958) However these compounds still await further structural elucidation

An increased titer of Porter Silber chromogens has been reported subsequent to an incubation of chloroform pre-extracted human plasma with commercial β glucuronidase (Bongiovanni and Eberlein 1955) In normal subjects given ACTH the plasma adrenal steroids liberated by hydrolysis with commercial β glucuronidase are likely to be tetrahydrocortisone and tetrahydrocortisol (Vermeulen 1956) Reduced metabolites of cortisol might therefore exist conjugated to glucuronic acid in human plasma whereas conjugated metabolites of aldosterone have hitherto not been described from this source By the technique of paper chromatography the blue tetrazolium reaction and the triphenyl tetrazolium reaction tetrahydro Compound A (3α 21-dihydroxy pregnane 11 20 dione) is present as a conjugated steroid in serum from children under ACTH stimulation (Klein *et al*

costeroids to such patients caused a fall in urinary 17 ketosteroids and a regression of virilizing symptoms (Wilkins *et al* 1951) On the basis of these and other studies the suggestion was made and subsequently verified that there might be some defect in the normal hydroxylation mechanisms which are concerned in the biosynthesis of hydrocortisone Thus it has been shown that there are increased quantities of pregnanetriol secreted in the urine of these subjects and that there appears to be a deficiency in the 21 hydroxylating mechanism (Jailer *et al* 1955) Such a defect in the synthesis of hydrocortisone would result in an increased secretion of ACTH with stimulation of adrenal androgen production and therefore production of the virilization which is seen in this syndrome A variation of this syndrome has been described in which there is a defect of both the 11 oxygenating and the C_{21} oxygenating systems This results in the production of increased quantities of Compound S (17 hydroxy 11 desoxycorticosterone) and 11 desoxycorticosterone as well as increased quantities of urinary androgens and the syndrome of hypertensive congenital hyperplasia is produced (Bongiovanni and Eberlein 1955b)

TABLE II Theoretical Extractability of Various Steroid Hormones Comparison of Efficiency of Chloroform and Ethyl Acetate Extraction

100 microgram Steroid	K chloroform/water	Microgram extracted from water by equal volumes of chloroform Extraction 1st	2nd	3rd	Total extracted (micrograms)
Cortisol	6.4	88.5	11.7	0.7	98.9
2α-Hydroxycortisol	0.3	23.0	17.8	13.7	54.5
6β Hydroxycortisol	0.05	4.8	4.5	4.3	13.6
	K ethyl acetate/water	Microgram extracted from water by equal volumes of ethyl acetate Extraction 1st	2nd	3rd	
Cortisol	12.2	92.5	6.9	0.6	ca 100
2α-Hydroxycortisol	1.9	65.5	23.3	7.4	96.2
6β Hydroxycortisol	0.9	47.3	24.8	13.1	85.2

Partition coefficient (Bernstein 1956)

unless the partition coefficient is very much in favor of the organic phase repeated extractions are necessary (Table II) The extraction method as outlined by Nelson and Samuels (1952) has found wide application Its disadvantage lies in the formation of emulsions However this can be avoided by keeping both the blood plasma and the organic solvent cool during extraction The ability of ethyl acetate to remove Porter Silber chromogens from plasma is greater than that of chloroform or methylene chloride (Brinck Johnsen and Eik Nes 1958) but repeated extraction with ethyl acetate will remove more plasma pigments than the other two solvents and evaporate more slowly in a partial vacuum at +40°C (Brinck Johnsen and Eik Nes 1958)

Axelrod and Zaffaroni (1954) have described extraction of adrenal steroids utilizing the principle of dialysis This method is particularly useful when the modifications of Lombardo *et al* (1955) and Nowaczynski and Genest (1955) are employed With these modifications the extraction of cortisol corticosterone and aldosterone from human plasma should be adequate (greater than 90 per cent) The extract obtained is very clean and can be applied to paper chromatograms without further purification

The method of charcoal absorption offers distinct advantages when large amounts of human blood are to be extracted This form of

1957) and in the single case of Eberlein and Bongiovanni (1956) conjugated tetrahydro Compound S was found in both plasma and urine

Thus this survey concentrates on chemical methods for the measurement of cortisol corticosterone aldosterone and the tetrahydrogenated metabolites of cortisol in human plasma Only a selection of publications can be mentioned briefly and space limitations do not permit detailed discussion

Biological tests for the determination of adrenal hormones have been developed the most sensitive being the detection of adrenal hormones influencing water and mineral balance in rats If one intends to assay adrenal steroids in human plasma these tests are of limited value since the plasma concentration of adrenal steroids is low However Vogt (1943) and Paschkis *et al* (1950) were able to demonstrate the presence of adrenocortical activity in the adrenal effluent of dogs by biological assays and as late as 1952 the preliminary work on aldosterone in adrenal vein blood from dogs and monkeys was done using biological criteria (Simpson *et al* 1952) In spite of the fact that *many of these biological tests were of immense importance for adrenal* physiology and pathology they are now of more historic interest since within the last ten to fifteen years chemical methods have been worked out for the estimation of small amounts of adrenal steroids in blood Such techniques involve three major analytical steps (1) extraction of steroids (2) purification of steroids and (3) identification and quantitative determination of steroids

EXTRACTION OF ADRENAL STEROIDS FROM HUMAN BLOOD PLASMA

The most commonly used method for extraction is the employment of organic solvents The selection of such solvents should not be a matter of convenience (Mason 1954) or what one has on the shelf but should be based on the partition coefficient for the particular adrenal steroid being determined (Burstein 1956) Table II shows the trouble one could encounter if 2α hydroxycortisol and 6β hydroxy cortisol were to be extracted without checking partition coefficients

Aldosterone corticosterone and cortisol can be extracted adequately from blood plasma with chloroform methylene chloride or ethyl acetate over a wide range of steroid concentrations One should extract the blood plasma with equal volumes of any of these solvents and

remove a large proportion of interfering material in the Porter Silber reaction (Eik Nes 1957) However a partition between benzene and water reduces the recovery of corticosterone considerably (Talbot *et al* 1945) This type of purification cannot be used if cortisol and corticosterone are to be determined in the same plasma sample Silber and Busch (1956) pre extract human plasma with carbon tetrachloride thereby achieving purification of the subsequent chloroform extract of such plasmas A partition between 70 per cent alcohol and hexane is a great help in cleaning blood extracts containing adrenal steroids (Farrell *et al* 1954)

Countercurrent distribution may have practical application for the separation of cortisol corticosterone and aldosterone in human blood Aldosterone has been adequately separated from cortisol in a 50 transfer countercurrent distribution at 21°C (Carstensen 1956) Carstensen claims that with 200 transfers the simultaneous separation of aldosterone from cortisone and cortisol would be possible with countercurrent distribution in a solvent system of 50 per cent 2 butanol and 50 per cent n hexane/water (Carstensen 1956) The separation of aldosterone from the other adrenal steroids in the plasma is relatively difficult and time consuming using paper chromatography (Farrell *et al* 1954)

Absorption chromatography and partition chromatography have been used in the techniques for detection of adrenal steroids in blood Adrenal steroids can be extracted from neutral alumina columns (Reichstein and Shoppee 1949) Nelson and Samuels (1952) introduced the use of magnesium silicate and Florisil columns for purification of Porter Silber chromogens in human blood plasma These columns were developed with chloroform and Porter Silber chromogens were extracted through elution with a mixture of ethanol and chloroform Further adrenal steroids can be effectively separated on silica gel columns (Pincus and Romanoff 1950) and micro silica gel columns (Sweat 1954a) Mattox and Mason (1956) found that cortisol and cortisone are rather unstable on a silica gel column and criticism has also been raised as to the stability of plasma Porter Silber chromogens on the Florisil column It is the experience of Samuels group that these plasma chromogens are stable on the Florisil column if the columns are properly prepared (Eik Nes *et al* 1953) However with the technique of Nelson and Samuels cortisol and corticosterone cannot be separated on the same column (Eik Nes and Bush 1958)

extraction should not be used indiscriminately since artifacts of the adrenal steroids can be produced (Meyer 1953)

The conjugated metabolites of cortisol in human plasma cannot be extracted with chloroform methylene chloride or ethyl acetate but can be extracted with ethanol or butanol If the dry residue from such extracts is dissolved in a buffer and hydrolyzed with β glucuronidase or acid the di and tetrahydrogenated metabolites of cortisol will be liberated from their conjugation and are soluble in chloroform (Bongiovanni and Eberlein 1955a) However the procedure employed by Edwards *et al* (1953) for conjugated urinary steroids should be explored for the extraction of plasma tetrahydrahydrocortisol and tetrahydrocortisone

Apparently the method of enzyme hydrolysis for steroid conjugates in blood is more efficient than that of acid hydrolysis (Klein *et al* 1957 Bongiovanni and Eberlein 1955b) Whereas plasma 17 ketosteroids exist conjugated to glucuronic acid (Migeon and Plager 1954) sulfuric acid (Bush 1958) and also phosphoric acid complexes (Oertel and Eik Nes 1958a) there has been no indication as yet that the di or tetrahydrogenated metabolites of cortisol in human plasma exist in any other form of conjugation than with glucuronic acid (Oertel and Eik Nes 1958b) Until complexes other than that of glucuronic acid are found in the plasma the use of commercial preparations of glucuronidase either from animal or bacterial sources might be considered an adequate hydrolysis One should bear in mind however that a rather crude enzyme preparation of β glucuronidase has been used for the splitting of a so called tetrahydrocortisol or cortisone glucuronide complex in blood (Bongiovanni and Eberlein 1955a Vermeulen 1956) and that the glucuronic acid part of this complex has been neither isolated nor identified

PURIFICATION OF ADRENAL STEROIDS IN EXTRACTS OF HUMAN BLOOD PLASMA

Considerable purification of an organic solvent extract containing the predominant plasma adrenal steroids can be achieved by shaking the organic extract with aqueous sodium hydroxide Acidic and phenolic substances present in the plasma extract are thereby separated from the adrenal steroids (Klyne 1957) This treatment transforms the lipid-soluble acids and phenols into their ionized sodium salts which are water soluble (Klyne 1957) Similarly a benzene wash of an aqueous solution containing plasma Porter Silber chromogens may

spectrophotometer the intensity of the light absorbed in the ultra violet range of the spectrum may yield information as to the amount of steroid present (Parke and Davis 1952 Eik Nes 1955)

When working with the conjugated metabolites of cortisol in blood plasma purification after enzyme hydrolysis can be achieved by sub jecting the hydrolyzate to dialysis (Brown *et al* 1957) The water phase from the dialysis is extracted with chloroform and the chloroform extract should be further purified on the Florisil column of Nelson and Samuels (1952) A ligroin water partition (Eik Nes 1957) before column chromatography is often useful (Brinck Johnsen and Eik Nes 1958) *A more adequate approach would probably be the* utilization of the chromatographic systems described by Schneider and Lewbart (1956) and by Bush (1957b) in which conjugated steroid complexes can be separated

IDENTIFICATION AND QUANTITATIVE DETERMINATION OF ADRENAL STEROIDS IN PURIFIED EXTRACTS OF HUMAN BLOOD PLASMA

There is no single method by which one can be sure that the quan titative determination of plasma cortisol corticosterone or aldosterone will be achieved Inasmuch as the measurement of these steroids in blood plasma is of clinical as well as of experimental importance a great many methods have been published which depend upon the ability of these steroids either to form chromogens with various re agents or to absorb light at certain wavelengths in the spectrum

It would seem logical to divide these methods into the following categories methods which are useful for screening purposes espe cially in the clinical laboratory and more refined methods which should be employed when one is interested in the quantitative as well as the qualitative determination of the steroid in question The latter methods require large amounts of normal blood plasma and the withdrawal of such volumes will cause a stress whereby the concentration of adrenal hormones in plasma *may increase*

In the subsequent evaluation of chemical methods for adrenal hormones in plasma this author has drawn from the experience of numerous investigators checking the different methods during training courses* in *steroid biochemistry*

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Partition chromatography with kieselguhr as supporting material (Butt *et al* 1951) might offer improvement over the adsorption chromatography (Klyne 1957) Bush (1954) believed this technique was equal in resolving power to that of partition chromatography *with filter paper*

The separation of different adrenal steroids in blood plasma extracts by partition chromatography with filter paper *as supporting material* has been applied successfully Valuable techniques of this type have been developed by Zaffaroni (1953) and by Bush (1952) Ascending chromatography preceding the descending chromatography on paper is highly useful especially when large volumes of blood plasma have been extracted or the plasma has been subjected to hydrolysis (Oertel 1956)

One should remember however *that the* techniques of paper chromatography should not be considered as a sole means of identification for any steroid A fairly constant running rate (R_f) can be found for the different steroids in different paper chromatographic systems but one can easily arrive at erroneous conclusions by using the running rates of steroids on paper chromatograms as criteria for their chemical nature (Bush 1954 Takeda 1956) Therefore only tentative conclusions should be arrived at using the R_f value of a spot on a paper chromatogram even if this spot runs like Compound F

An advantage of paper chromatography is that reactions are known which permit identification of steroids directly on the paper Adrenal hormones having a Δ^4 3 keto arrangement in ring A of the steroid nucleus can be detected on the paper strip by an ultraviolet scanning technique (Haines and Drake 1950) If a paper chromatogram is sprayed with tetrazolium salts steroids with a primary α ketol side chain will reduce these salts to colored formazans (Zaffaroni 1953) *The soda reaction of Bush can be applied directly to paper strips* and will localize Δ^4 3 ketosteroids (Bush 1952) Recently isonicotinic acid hydrazide dissolved in hydrochloric acid has been shown to give a yellow green fluorescence under ultraviolet illumination with Δ^4 3 ketosteroids on paper chromatograms (Weichselbaum and Margraf 1957) and the m dinitrobenzene and 2 4 dinitrophenylhydrazine reactions have been used to detect adrenal steroids on paper strips *(Kochakian and Stidworthy* 1952) The sensitivity of these methods for qualitative estimation of adrenal hormones on paper strips has been discussed by Bush (1954) Furthermore by passing a paper chromatogram containing a Δ^4 3 ketosteroid through a microslit in a

Nes (1957) and Peterson *et al* (1957) have published short cut methods for the detection of plasma 17 hydroxycorticosteroids These methods are less time consuming than the original method of Nelson and Samuels In normal human subjects plasma concentrations of Porter Silber chromogens from 0 to 27 μg per hundred milliliters can be found (Table III) The method of Peterson *et al* (1957) and that of Silber and Porter (1954) with a pre-extraction of the plasma with carbon tetrachloride (Silber and Busch 1956) seem to be very reliable for the clinical laboratory However in cases where a dysfunction of the liver is present the technique of Eik Nes (1957) may be preferable

In the Porter Silber reaction the side chain of the cortisol molecule is primarily involved in the reaction (Wilson and Fairbanks 1954 Bongiovanni and Eberlein 1955a) Hence one should be very careful in evaluating the chromogen spectrum formed (Fig 2) Unless this spectrum is measured from 370 to 450 millimicrons and a peak absorbance is found at 410 millimicrons plasma concentration of

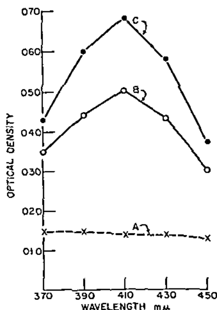


FIGURE 2 Average optical density at five different wavelengths of Porter Silber *chromogens* extracted from dog plasma (*A*) 10 ml of hypophysec tomized dog plasma obtained between the fourth and the thirtieth day after hypophysectomy (*B*) 10 ml of normal dog plasma obtained between 8 A M and 11 A M (*C*) 0.5 μg of cortisol taken through the total procedure for the determination of plasma Porter Silber chromogens (Eik Nes 1957) (From Eik Nes K and Brizzee K R *Am J Physiol* 193 403 1958)

Porter and Silber (1950) observed that when 17 21 dihydroxy 20 ketosteroids were treated with phenylhydrazine in sulfuric acid a yellow color was produced with a maximal absorbance at 410 milli microns Utilizing this color reaction Nelson and Samuels (1952) published a method for the detection of 17 hyroxycorticosteroids in human plasma Numerous published experiments have employed this method which seems to be reliable (Harwood and Mason 1956) Few naturally occurring substances in plasma interfere with the formation of the Porter Silber chromogen if the plasma extracts are properly purified (Eik Nes 1957) Also the results obtained with this method correspond to those of more elaborate procedures for the detection of plasma cortisol (Migeon *et al*, 1956b Eik Nes 1955) Using the phenylhydrazine sulfuric acid reaction Silber and Porter (1954) Eik

TABLE III Adrenal Steroids in Systemic Blood of Normal Human Subjects

A th	Re ti	Mi og am /100 ml f blood pl m		
		Co t l	C rtico t ro	Co ti l/ C ti ost ro *
Nelso and Sam els (1952)	(PS)	4 10[†]		
Bliss et al. (1953)	(PS)	3 24		
Bayli s a d Steinbeck (1953)	(PS)	ca 10		
Ely et al. (1953)	(PS)	0 27		
Kass nar et al (1958)	(PS)	3 14		
Silbe and Porter (1954)	(PS)	6 25		
Peter et al (1957)	(PS)	6 25		
Bo dy and Altr ck (1953)	(PS)	3 14		
Eik Ne (1957)	(PS)	6 18		
Sweat (195)	(F)[‡]	10 8 (X)[§]	4 3 (X)	2 5
W lchs lbaum and Margraf (1955)	(PS) and Made and B ck (1952)	9 5 (X)	8 9 (X)	1 7
Pete o (1957a)	(F) (P)	6 25	0 5 2 0	ca 13 0
Morris a d William (1953a,b 1955)	Pol ographic stim t on	5 13	4 16	ca 1 0
B sh and Sandbe g (1953)	N mero reactions	ca 10	ca 1 5	4 0
Starlinge and Tamm (1955)	(F)	6 18	1 4 5 5	a 3 0
Bo dy d Upton (1957)	*(F)*	4 17	0 5	c 8 0
Lewi (1957)	(F)	6 12	0 6	a 8 0
Takeda (1956)	(F)	9 2 (X)	6 1 (X)	a 1 5
Ely t al (1958)	(PS) (F)	5 16	2 2 5 2	a 3 6
Silb r t al (1958)	(F)	23 (X)	1 8 (X)	13 0

(P*) Porter-Silb tion f cortisol
† Whol blood
‡ (F) Fluo nc
§ (X) Mean c tr ti

chromatography of a derivative of the unknown sample Since the plasma concentration of adrenal steroids is low the technique of preparing a radioactive acetate may be preferable on many occasions (Luetscher *et al* 1955 Berliner 1956) Additional information can be gained utilizing the principle of isotope dilution (Rittenberg and Foster 1940 Berliner and Salhanick 1956 Peterson 1957b) If for final proof of identification measurement of the sulfuric acid spectrum (Haines and Drake 1950) micro infrared spectrophotometry (Plantin 1955) or a micro scale chemical transformation (Zaffaroni 1953) is to be employed the acetyl group can be removed by treating the compound with choline esterase as elegantly demonstrated by Farrell *et al* (1954) Using this technique we have recently converted cortisone 4 C^{14} acetate to cortisone-4 C^{14} in better than an 80 per cent yield (Fevold and Eik Nes 1958)

A considerable difference exists regarding concentrations of cortisone in the systemic circulation of man (Table III) Using the method of Sweat a plasma ratio of cortisol/corticosterone is found to be 2 to 4 Takeda (1956) in a criticism of this method thinks that nonsteroid material may fluoresce By the method of Morris and Williams (1953b 1955) the ratio of cortisol/corticosterone in blood plasma is about 1 Peterson (1957a) has contributed valuable data explaining why one would expect this ratio to be at least 10 in normal human plasma The methods published by Bondy and Upton (1957) and by Peterson (1957a) for the determination of corticosterone in plasma seem to be extremely well designed Another promising approach to the problem of plasma corticosterone is the sulfuric acid fluorescence method of Silber *et al* (1958) When the identification of plasma corticosterone is the object of investigation the procedures of Bush and Sandberg (1953) combined with further chemical tests as outlined for the identification of plasma cortisol are suitable The minute concentration of corticosterone in peripheral human blood however requires large amounts of blood for such techniques (Peterson 1957b)

Methods for the chemical estimation of aldosterone in urine have been developed (Luetscher *et al* 1955) but such methods have hitherto not been available for human blood plasma Recently Kliman and Peterson (1958) published a method whereby plasma aldosterone can be adequately determined This method makes use of double labeling and employs two paper chromatograms In spite of the expense and the time involved in such an analytical procedure it should be of great value in both the experimental and clinical laboratory

Porter Silber chromogens should be considered valueless The concentration of Porter Silber chromogens in the unknown plasma samples should be calculated from the recovery of small amounts of cortisol or cortisol C^{14} taken through the entire analytical procedure

Plasma substances giving rise to a positive Porter Silber chromogen spectrum have often been designated 17 hydroxycorticosteroids (17 OHCS) in the literature In spite of the fact that cortisol is the predominant adrenal hormone in the systemic circulation of man (Bush and Sandberg 1953 Hudson and Lombardo 1955 Sweat 1955) it is a question whether material measured by the sulfuric acid phenyl hydrazine reaction should not be called Porter Silber chromogens (Borth 1956)

Sweat (1954a) makes use of a silica column for the purification of blood extracts and the measurement of sulfuric acid-induced fluorescence of cortisol and corticosterone (Sweat 1954b) This method employs less plasma than the Nelson Samuels method (1952) and apparently is well suited for cortisol determination (Takeda 1956) Recently Silber *et al* (1958) utilizing the sulfuric acid fluorescence of cortisol have developed a method for the detection of this hormone in small volumes of human plasma This seems to be a very promising technique for the clinical laboratory

For more refined purposes cortisol can be determined as outlined by Bush and Sandberg (1953) Since it has been observed that some of the pyrimidine bases in plasma may have an R_f on paper chromatograms close to that of cortisol (Harris and Eik Nes 1958) and corticosterone (Takeda 1956) and also absorb ultraviolet light it is necessary to perform elution of the paper chromatograms and to determine in a spectrophotometer the maximal ultraviolet absorbance of material with an R_f value of synthetic cortisol In elution of paper chromatograms with ethanol or methanol nonsteroidal material is also removed with the alcohol We have found that if this alcohol extract is evaporated in nitrogen dissolved in methylene chloride and washed with $1/10$ volume of 0 1 N HCl a considerable purification of cortisol and corticosterone is achieved in the methylene chloride phase (Oertel and Eik Nes 1958b) Also chromatography on micro silica gel columns is helpful in removing impurities extracted with alcohol from paper strips (Brinck Johnsen and Eik Nes 1957)

To establish further the chemical nature of adrenal steroids present in purified extracts from paper chromatograms rechromatography in another system is necessary followed by mixed chromatograms and/or

The Influence of Extra Adrenal Factors on Blood Corticosteroids

Avery A Sandberg

The blood corticosteroid levels depend on three important factors (1) supply (adrenocortical secretion) (2) distribution (exchange between the intravascular and extravascular fluids and tissues) and (3) disposal The role of the first factor has been discussed in Section 1 of this chapter The present section is concerned with the remaining two factors both essentially extra adrenal in origin

A knowledge of the fate of cortisol and the methods used for measuring it is indispensable for an understanding of the extra adrenal factors influencing the concentration of corticosteroids in blood Deliberate emphasis is placed on cortisol since it constitutes the preponderant corticosteroid secreted by the human adrenal cortex (Romanoff *et al* 1953 Hudson and Lombardo 1955 Hardy and Turner 1957) and since it has been found to be the major corticosteroid in the peripheral blood (Bush and Sandberg 1953 Sweat *et al* 1953) Additionally in contrast to cortisol until recently no efficient or practical method was available for the measurement of the concentration of corticosterone the other important human adrenal corticosteroid in small samples of blood (Bondy and Upton 1957 Peterson 1957a b Ely *et al* 1958 Silber *et al* 1958)

The major steps in the metabolic transformation of cortisol (I) are shown in Figure 3 (Dorfman and Ungar 1953) Step (a) the most important with respect to metabolic activity of cortisol itself and of subsequent compounds involves the reduction of the double bond in ring A with the production of dihydrocortisol (II) Existing evidence suggests that step (a) is rate limiting in the metabolism of cortisol in the body and that the enzyme system involved may be specific for cortisol (Dorfman and Ungar 1953 Peterson *et al* 1955 Peterson and Wyngaarden 1956 Tomkins 1956) Further reduction of ring A occurs by hydrogenation of the ketone (step b) with the production of tetrahydrocortisol (III) Even though the processes involved in the transformation of tetrahydrocortisol (III) to cortol (IV) may occur prior to step (d) the final result is the formation of a glycerol side chain from the dihydroxyacetone side chain (carbons 17 20 and 21) A small amount (1 to 2 per cent) of cortisol (I) is transformed to a

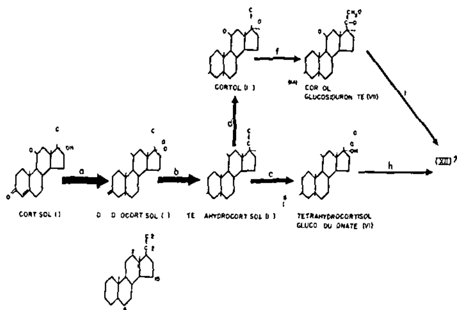
The determination of Porter Silber chromogens in blood plasma after hydrolysis with β glucuronidase has been described (Bongiovanni and Eberlein 1955a) This method has found its proper place in the clinical laboratory and reproducible results can be obtained The amount of conjugated Porter Silber chromogen in the plasma should be calculated from the recovery of synthetic tetrahydrocortisol or tetrahydrocortisone (Wilson and Fairbanks 1954) as done for the detection of these reduced metabolites in the urine (Romanoff *et al* 1957) For the identification of tetrahydro compounds in the plasma the chromatographic system of Schneider and Lewbart (1956) or of Bush (1957b) or electrophoretic separation of conjugated steroids on paper (Voigt and Beckmann 1953) might be promising followed by cleavage of the plasma complex with an enzyme and the use of previously mentioned microchemical methods for the identification of steroid hormones One should also strive for the identification of the material conjugating tetrahydrocortisol and tetrahydrocortisone in human blood plasma The fact that subsequent to hydrolysis with commercial β glucuronidase acid hydrolysis liberates additional steroid hormones in biological extracts does not indicate that the material hydrolyzed by acid is a sulfate

In summary chemical methods are available for the detection of the predominant adrenal hormones in small volumes of human plasma However these are micromethods and must be handled with extreme care One should certainly be aware of the limitations of these methods and identification of any of the predominant adrenal hormones from human plasma based on few criteria should be earmarked as such

17 ketosteroid (V) by cleavage of the side chain (Dorfman and Ungar 1953 Sandberg *et al* 1957c) It should be pointed out that all the metabolites of cortisol mentioned so far are essentially inactive bio logically In addition all the compounds (I to V) can be extracted from plasma with organic solvents (chloroform methylene chloride ethyl acetate or dichloromethane) and constitute the *unconjugated* steroids The next steps (c f or g) in the metabolism of the cortisol derivatives (III to V) involve conjugation primarily with glucuronic acid (VI to VIII) and to a lesser extent with other substances possibly including sulfuric acid These *conjugated* metabolites (VI to VIII) are more water soluble than the unconjugated ones and cannot be ex tracted with the solvents mentioned above unless the glucuronic or sulfuric acid is first hydrolyzed off with β glucuronidase or acid respec tively (Bongiovanni 1954 Klein *et al* 1955) The conjugates can be extracted from the plasma however with aqueous ethanol or butanol Further metabolism (steps h i and j) of the cortisol derivatives occurs The nature of these metabolites and their conjugates (XII) remains unknown It should be pointed out that the transformation of cortisol to hitherto unknown metabolites may occur prior to the steps indi cated (h i and j) but for simplicity have been presented as they are in Figure 3 One important metabolic step consists of the oxidation of the hydroxyl group at carbon 11 resulting in the formation of tetrahydrocortisone (IX) instead of tetrahydrocortisol (III) and of cortolone (X) instead of cortol (IV) both major metabolites of cortisol found in the urine In addition although the reduction of the side chain to a glycerol (IV) is shown to result in the α position of the hydroxyl group at carbon 20 (H—C—OH) a substantial amount is converted to the β form (HO—C—H) (Fukushima *et al* 1955)

Most of our knowledge regarding the extra adrenal factors influ encing the blood levels of the corticosteroids is based on one of two methods for the analysis of cortisol and its metabolites Although other useful methods for the determination of cortisol and some of its metabolites have been published (Freeman *et al* 1956 Bondy *et al* 1957) they have not been used as extensively as the methods to be discussed

The most efficient practical and widely used chemical methods for the estimation of cortisol and those of its metabolites in the blood



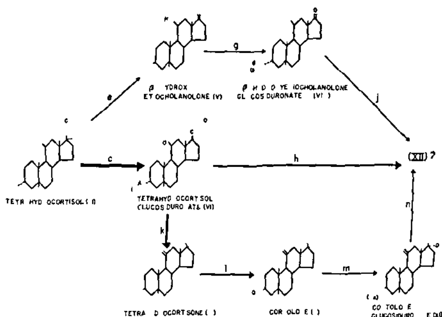


FIGURE 3a and 3b Schematic and simplified presentation of the pathways involved in the metabolism of cortisol The thickness of the arrows indicates roughly the quantity of steroid metabolites involved in the metabolic path way GA glucuronic acid SA sulfuric acid

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retaining the dihydroxyacetone side chain (17 hydroxycorticosteroid) are based on the Porter Silber reaction (Porter and Silber 1950 Nelson and Samuels 1952 Eik Nes 1957) It has been shown that in normal plasma or plasma obtained from subjects following the administration of cortisol about 90 per cent of chloroform or dichloromethane extractable Porter Silber chromogens is cortisol (Peterson and Wyngaarden 1956 Peterson *et al* 1955 1957 Migeon *et al* 1956b) On the other hand following β glucuronidase hydrolysis and extraction with chloroform or dichloromethane the preponderant part of the Porter Silber reacting material is in the form of tetrahydrogenated compounds (III and IX) It is apparent from an examination of Figure 3 that many metabolites of cortisol are not determined by the Porter Silber reaction and changes in the levels of 17 hydroxy corticosteroids in the blood may merely be a reflection of changes in the ratios of the various metabolites of cortisol rather than an indication of quantitative changes in cortisol levels

The synthesis and availability of C^{14} labeled cortisol has made possible a study of the metabolism of this steroid with near tracer amounts Following the injection of cortisol C^{14} only 50 per cent of the unconjugated radioactivity in the plasma is due to cortisol C^{14} (Peterson *et al* 1955 Peterson and Wyngaarden 1956) Because the Porter Silber reagent reacts only with the 17 21 dihydroxy 20 keto side chain this indicates the failure of the method to measure a substantial part of the unconjugated cortisol metabolites The same applies to the metabolites in the glucosiduronate fraction

The recoveries from urine of cortisol metabolites following the administration of cortisol differ with the method used for the measurement of the steroids in the urine Following injection of large amounts of cortisol employing a method based on the Porter Silber reaction and β glucuronidase hydrolysis only about 30 per cent of the injected steroid can be accounted for in the urine in 48 hours with 2 to 3 per cent in the unconjugated fraction (Sandberg *et al* 1953a b Sayers *et al* 1955) On the other hand using radioactive measurements following cortisol C^{14} injection in small amounts 90 per cent of the injected radioactivity is excreted in the urine in 48 hours (Peterson and Wyngaarden 1955 1956 Peterson *et al* 1955 Hellman *et al* 1954 1956 Migeon *et al* 1956c Samuels 1957 Samuels *et al* 1957) About 50 to 60 per cent of the metabolites are found in the glucosiduronate fraction 10 to 15 per cent in the acid hydrolyzed fraction and 5 to 10 per cent of the radioactive metabolites can be released by

hydrolysis with hot acid Only 3 to 4 per cent of the radioactivity appears in the unconjugated fraction in the urine primarily within the first 4 hours following the injection of cortisol C^{14} Only about 5 per cent of the injected radioactivity of cortisol-C^{14} appears in the bile most of it ultimately finding its way into the stools

The points outlined above are important in the understanding and evaluation of the metabolism of cortisol under various conditions Since in many of the published studies no attempts were made to identify rigidly the Porter Silber chromogens or the radioactive metab olites the interpretation of such investigations has to be tempered by the possibility that the unusual preponderance of various path ways of cortisol metabolism rather than a change in its rate of metabolism may be responsible

The extra adrenal factors to be considered in the following sections are primarily concerned with the effects of distribution and disposal on the level of blood corticosteroids These extra adrenal factors may influence the concentration by affecting equilibration between the vascular and extravascular compartments by influencing the distri bution between tissues and extracellular fluids or by direct effect on those structures which are concerned with the removal of cortisol from the body Additionally extra adrenal factors may influence the rates of the various enzymatic steps involved in the metabolism of cortisol Extra adrenal factors are of special importance following the administration of large doses of cortisol These factors may be related to the concentration of substrate and the effects of administered cortisol on certain enzymatic reactions

THE RATE OF METABOLISM AND THE MISCIBLE POOL OF CORTISOL

Much has been learned regarding the metabolism of cortisol under normal and abnormal conditions (Bondy and Altrock 1953 Perkoff *et al* 1954 Bongiovanni and Eberlein 1955a Plager *et al* 1957) Following the intravenous injection of large doses of cortisol measure ments of the theoretical volume of distribution of the injected cortisol and in conjunction with the response of the plasma 17 hydroxycorti costeroids to maximal amounts of ACTH (Eik Nes *et al* 1954 1955a) the rate of cortisol secretion has been calculated (Samuels *et al* 1957) Even though the doses injected were unphysiological the changes in the plasma 17 hydroxycorticosteroids have been used as a

useful measure of the rate at which exogenous cortisol is metabolized under various conditions

The synthesis and availability of cortisol C^{14} in near tracer amounts has made possible the measurement of the rate of metabolism of cortisol its miscible pool and its turnover in a more elegant physiological and accurate way at nearly physiological concentrations of the steroid (Peterson *et al* 1955 Migeon *et al* 1956c Peterson and Wyngaarden 1956) The description of the conditions as nearly physiological following the injection of cortisol C^{14} stems from the fact that although the amount of carrier steroid injected may not significantly raise the plasma 17 hydroxycorticosteroid levels using presently available methods for their determination it does increase the miscible pool of cortisol by as much as 25 per cent

The concentration of cortisol in plasma is dependent on the size of the miscible pool in which it is distributed and the turnover rate of this pool (Benedict *et al* 1949 Peterson *et al* 1955 Peterson and Wyngaarden 1956) After the injection of cortisol C^{14} the unconjugated steroids show a rapid initial fall over a period of 20 to 60 minutes (Fig 4) Following this rapid fall the concentration in the

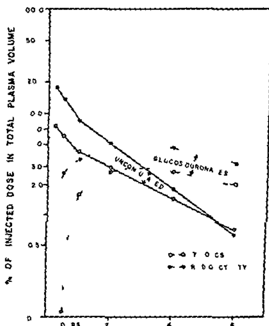


FIGURE 4 Plasma unconjugated and conjugated (glucosiduronated) 17 OHCS and radioactivity following the injection of cortisol The plasma 17 OHCS were obtained following the intravenous injection of 70 mg of cortisol into a normal male subject weighing 70 Kg The radioactivity was obtained fol

plasma over the next 6 to 8 hours falls on a logarithmic curve of lesser slope when plotted against time This exponential decay is interpreted as indicating equilibrium between the rate of destruction of cortisol and the rate of adrenocortical secretion and has been used as an indication of the rate of metabolism of endogenous cortisol Extrapolation to zero time of the more gradual curve gives an indication of the size of the miscible pool of cortisol its biological half life and the rate of its turnover (see Fig 4) On the basis of curves obtained with cortisol C^{14} the miscible pool averages 1 8 mg with half life of 1 hour (Peterson *et al* 1955 Migeon *et al* 1956c Peterson and Wyngaarden 1956 Plager *et al* 1957) These figures are consonant with current knowledge of the amount of cortisol secreted under physiological conditions by the human adrenal cortex as determined by the amount of cortisol metabolites present in the 24 hour urine of normal subjects

Calculations of the turnover rate of cortisol reveal an average value of approximately 25 mg per day Calculation of the total 24 hour secretion of cortisol on the basis of the turnover of the miscible pool

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lowing the intravenous injection of cortisol 4 C^{14} (1 μC/250 μg 1 500 000 c/m) The possible causes for the differences in the clearance rates and concentrations between the two methods are discussed in the text

The miscible pool in this subject can be calculated from the cortisol C^{14} unconjugated curve The calculations are based on assumptions the validity of which has been discussed in the original articles (Benedict *et al* 1949 Peterson *et al* 1955 Peterson and Wyngaarden 1956)

$$A = a\left(\frac{I}{I_0} - 1\right)$$

A miscible pool a = amount of cortisol injected
I specific activity of the injected steroid
I_0 specific activity of the miscible pool immediately after injection

This subject had a 17 OHCS level of 14 μg per hundred milliliters at the time of injection of the cortisol C^{14} Extrapolation of the lower portion of the unconjugated curve to zero time reveals the plasma (and probably *the miscible pool) to have a specific activity ten times less than that of the* injected steroid Substitution of the figures obtained in the formula reveals that the miscible pool of this subject was 2.25 mg

The biological half time of the cortisol C^{14} can be obtained from the linear plot of the lower portion of the curve In this subject the half time ($t_{½}$) is approximately 80 minutes Since the lower portion of the curve can be shown to follow a first order reaction the negative slope of the curve will *give the turnover rate of the pool*

$$\text{Turnover rate } (K) = \frac{\log 2}{t}$$

Substitution of the appropriate figures in the formula will indicate that the above subject turned over 0 52 of his miscible pool per hour

The half time of the nonradioactive unconjugated 17 OHCS (open circles) in the plasma *following the administration of 50 mg of cortisol intravenous* is about 2 hours This is obtained by extrapolation of the curve to zero time

may be spuriously high or low depending on the time of day when the determination is performed It has been shown (see p 373) that the concentrations of the plasma 17 hydroxycorticosteroids and the miscible pool of cortisol exhibit a diurnal variation due to changes in the adrenocortical secretory activity (Bliss *et al* 1953 Peterson *et al* 1955 Migeon *et al* 1956a Peterson and Wyngaarden 1956) Following the intravenous administration of large amounts of cortisol (50 mg or more) there is an initial rapid fall of the unconjugated 17 hydroxy corticosteroids followed by decreasing concentrations of the 17 hydroxycorticosteroids in the plasma over the next 6 to 8 hours (Eik Nes *et al* 1955b Brown *et al* 1957 Scheuer and Bondy 1957) These values also fall on a logarithmic curve when plotted against time indicating removal of a constant fraction per unit time (Samuels *et al* 1957) The slope of the cortisol removal curve as expressed by the decline in the plasma 17 hydroxycorticosteroid levels has been used as an index of the rate of metabolism of cortisol under normal and abnormal conditions The half time of disappearance of the unconjugated 17 hydroxycorticosteroids following the injection of cortisol is on the average approximately 2 hours (Fig 4) When plotted the slope of the 17 hydroxycorticosteroid curve is much less than that of the radioactive steroids following cortisol C^{14} injection It should be stressed however that the amounts of cortisol administered are greatly in excess of those normally present in the body or produced by the adrenal cortex The half time obtained from these slopes represents predominantly the rate of *disappearance* of cortisol from the plasma and probably from the miscible pool On the other hand the half time obtained with near tracer doses of cortisol C^{14} as measured by the decline of the specific activity of the plasma cortisol is markedly influenced by the rate of *appearance* of newly synthesized unlabeled cortisol by the adrenal cortex (Peterson *et al* 1955 Peterson and Wyngaarden 1956) It should be reiterated however that the amount of cortisol C^{14} plus carrier injected falls short of being a true tracer dose (0 3 mg or more) This amount may affect cortisol synthesis or metabolism either by inhibition of ACTH release or by a significant increase in the miscible pool as is the case following the injection of large amounts of cortisol

The initial rapid fall in unconjugated steroids following the administration of either a large dose of cortisol or near tracer amounts of cortisol C^{14} has been assumed to relate to the distribution and equilibration of the injected cortisol between the vascular and extravascu

lar compartments Several intravascular factors primarily related to the plasma may influence this distribution and equilibration of cortisol and its metabolites As will be discussed (see p 496) there is evidence which indicates the existence of a protein (transcortin) in human plasma which has great affinity for cortisol (Bush 1957a Sandberg *et al* 1957b Daughaday 1958a b Slaunwhite and Sandberg 1959) At physiological concentrations of cortisol over 90 per cent is bound to transcortin Even though transcortin binds cortisol much more avidly than any of the other known plasma proteins a state of equilibrium exists between transcortin and the other plasma proteins which bind cortisol to a smaller extent The most important protein from a quantitative view in the latter group is albumin It is postulated that once the binding capacity of transcortin is saturated some extravascular tissues compete with the other plasma proteins for cortisol binding (Slaunwhite and Sandberg 1959) Calculation of the miscible pool of cortisol in the body indicates that considerably more than 50 per cent of it is located extravascularly Accordingly one must suppose a higher affinity for cortisol by some tissues than that shown by plasma Until more is known about the concentration and role of transcortin under various physiological and pathological conditions one can only speculate that it may have major importance in regulation of cortisol metabolism The same statement applies to the tissue binding of cortisol

Following the intravenous injection of large or near tracer doses of cortisol the unconjugated steroids are cleared from the plasma at an initial rapid rate which is related to distribution of the injected cortisol between the vascular and extravascular compartments The subsequent slower rate is related to the metabolism of the injected or endogenous cortisol It is apparent that these two processes (distribution and disposal) overlap and the resultant curves are greatly affected by the factors which determine each curve individually

Even though the theoretical or apparent distribution volume following the injection of cortisol has no anatomical reality (Peterson *et al* 1955 Peterson and Wyngaarden 1956 Samuels *et al* 1957) since in many studies it exceeds not only the extracellular fluid volume but also the total body water it has proved useful in the hands of some investigators in the evaluation of cortisol metabolism (Samuels *et al* 1957) After the administration of large amounts of cortisol the theoretical distribution volume of cortisol (at concentrations equal to those in plasma at the time of observation) is approximately 50 to 70

liters Following cortisol C^{14} the distribution volume may be as high as 25 to 35 liters Both these values seem unrealistic In either case it is apparent that only a small portion of the cortisol is left in the blood and that the major portion is taken up by tissues That all the cortisol is not distributed in the extravascular body water is demonstrated by the very low concentrations of the steroid in body fluids (spinal fluid ascites etc) however high concentrations of 17 hydroxycorticoster oids can occur in these sites in cases of inflammation of the surfaces involved (peritonitis meningitis etc) (Sandberg *et al* 1954b) The absence of cortisol in noninflammatory body fluids and its presence in protein containing exudates is consonant with the binding of the steroid to transcortin and other proteins

The discrepancies in the slopes of the unconjugated 17 hydroxy corticosteroids and of the radioactivity as determined by the 17 hydroxycorticosteroid method and by the radioactivity technique respectively bear explanation Aside from methodological and physio logical differences evidence has been presented that the rate of metab olism of cortisol is also influenced by the amount of steroid injected (Samuels *et al* 1957) It would appear that if the amount of steroid injected exceeds the binding capacity of transcortin and other plasma proteins a much larger portion of the cortisol finds its way into extra vascular components and tissues This results in a relatively lower plasma concentration of 17 hydroxycorticosteroids and a spuriously increased miscible pool (or theoretical volume of distribution) On the other hand following the injection of a near tracer dose of cortisol C^{14} a relatively greater portion remains in the plasma due to the avid binding and capacity of transcortin with the result that the calculated miscible pool (and theoretical volume of distribution) is much smaller Even with near tracer doses it is possible to exceed the binding capac ity of transcortin depending on the endogenous 17 hydroxycorticos teroid levels Some evidence for this view exists in the appearance of unconjugated steroid in the urine primarily within the first 4 hours following the injection of cortisol C^{14} Increasing amounts of uncon jugated steroids have been shown to appear in the urine of subjects injected with increasing amounts of cortisol Additionally it has been shown that both the zero intercept and the slope of the curve of the disappearance of unconjugated radioactivity following the injection of a near tracer dose of cortisol C^{14} depend on the initial concentra tion of cortisol in the plasma (Samuels *et al* 1957)

Most of the studies on the metabolism of cortisol have been based on the determination of its concentration in plasma Considerable confusion continues to exist regarding the amount of cortisol associated with the red blood cell Some workers feel that at physiological levels very little of the corticosteroids or their metabolites are associated with the erythrocytes although there are some indications that at high or nearly physiological levels of cortisol substantial amounts (18 to 36 per cent) may be associated with the red blood cells (Peterson *et al* 1955 Peterson and Wyngaarden 1956 Migeon *et al* 1959) This amount exceeds that which can be ascribed to the trapped plasma Even though there is some evidence to the contrary it would appear that under physiological conditions the preponderant part of the 17 hydroxycorticosteroids is present in the plasma but significant amounts may be associated with the erythrocytes No conjugated 17 hydroxycorticosteroids were found to be associated with the red blood cells (Migeon *et al* 1959) *In vitro* studies have shown that erythrocytes bind nearly 10 per cent of cortisol C^{14} when the labeled steroid is added to the blood (Sandberg *et al* 1957) Very little is known about the concentrations of 17 hydroxycorticosteroids in the leukocytes and platelets of the blood

Diurnal Variation

Important in the study of the metabolism of cortisol is the realization that a diurnal variation exists in the levels of the steroid a finding which also applies to the 17 hydroxycorticosteroids of the urine The factors controlling this variation have not been established That the diurnal changes in the 17 hydroxycorticosteroids are related to changes in adrenocortical production and to changes in the size of the miscible pool at different times of the 24 hours have been demonstrated (Peterson *et al* 1955 Peterson and Wyngaarden 1956) Whether the diurnal variations in the plasma 17 hydroxycorticosteroid levels are accompanied by changes in the rates of other types of cortisol metabolism remains to be determined The highest concentration of plasma 17 hydroxycorticosteroids occurs between 4 a m and 8 a m followed by a gradual decline until noon when the levels become relatively stable until midnight (Fig 5) After midnight the concentrations start rising again It has been shown that the diurnal variation does not show any deviation from the one shown in Figure 5 in night workers or in blind subjects A possible central nervous system regulation of the diurnal

liters Following cortisol C^{14} the distribution volume may be as high as 25 to 35 liters Both these values seem unrealistic In either case it is apparent that only a small portion of the cortisol is left in the blood and that the major portion is taken up by tissues That all the cortisol is not distributed in the extravascular body water is demonstrated by the very low concentrations of the steroid in body fluids (spinal fluid ascites etc) however high concentrations of 17 hydroxycorticosteroids can occur in these sites in cases of inflammation of the surfaces involved (peritonitis meningitis etc) (Sandberg *et al* 1954b) The absence of cortisol in noninflammatory body fluids and its presence in protein containing exudates is consonant with the binding of the steroid to transcortin and other proteins

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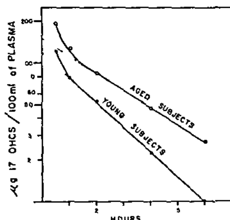


FIGURE 6 Disappearance of 17 OHCS from the plasma following the injection of cortisol (1 mg per kilogram of body weight) into aged and young subjects Extrapolation of the slow component to zero time reveals a half time of less than 2 hours for the young subjects and approximately 2½ hours for the aged subjects Note slower equilibration of the injected cortisol in the aged subjects The data presented in the figure represent a composite of results previously published (Tyler *et al* 1955 Samuels *et al* 1957)

slower than adults and that the rate of metabolism is inversely proportional to the age and surface area in childhood (Ely *et al* 1953)

The plasma 17 hydroxycorticosteroid levels in newborn infants tend to be much lower than those in the plasma of the mothers (Gemzell 1954 Klein *et al* 1954 Migeon *et al* 1955 1956d) Thus in one of the studies the concentrations of 17 hydroxycorticosteroids in cord plasma were approximately 20 to 50 per cent of those of the mothers in cases of vaginal delivery and 20 per cent or less in cases of mothers undergoing Caesarian section Some of the studies indicated that near gestation cortisol can pass through the placental barrier the 17 hydroxy corticosteroid concentration in the maternal blood being the determining factor as to the amount of the 17 hydroxycorticosteroids under going passage (Migeon *et al* 1956d) It would appear that the fetal adrenal gland produces little or no 17 hydroxycorticosteroids Recently it has been shown that newborn infants turn over administered large doses of cortisol more slowly than do children (Bongiovanni *et al* 1958 Grumbach) although the rates indicated by Bongiovanni *et al* (1958) are probably too low due to the administration of the hemisuccinate of cortisol rather than the free alcohol of the steroid (Grumbach) Of interest is the demonstration that the transcortin levels in the plasma of newborn infants are much lower than those in the plasma of the mothers (Sandberg and Slaunwhite 1959) The

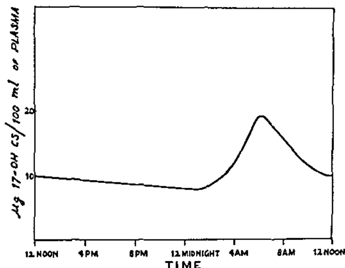


FIGURE 5 Diurnal variation of plasma 17 OHCS in a group of normal subjects

changes in the 17 hydroxycorticosteroids has been suggested in a recent study on subjects with severe brain damage (Eik Nes and Clark 1958)

It has been recently demonstrated that a diurnal variation very similar to that of cortisol appears in the plasma concentrations of corticosterone (Peterson 1959)

Effects of Age

Even though the plasma 17 hydroxycorticosteroid levels in subjects over 65 years of age are within the normal range these subjects clear the unconjugated 17 hydroxycorticosteroids at a slower rate than the subjects under 55 (Fig 6) (Tyler *et al* 1955 Samuels *et al* 1957) There is also some indication that the initial rapid rate of distribution is slower in aged subjects so that equilibrium is achieved somewhat later The extrapolation of the decay curve of the 17 hydroxycorticosteroids indicates a moderately smaller miscible pool which in conjunction with the normal 17 hydroxycorticosteroid levels would seem to indicate a reduced rate of secretion by the aged adrenal cortex The exact reasons for the decreased rates of 17 hydroxycorticosteroid production distribution and metabolism in aged subjects remain unknown

The plasma 17 hydroxycorticosteroid levels in infants and children are of the same magnitude as those found in the plasma of adult subjects There is some evidence that children metabolize cortisol

corticosteroid from the plasma following the administration of large amounts of cortisol or near tracer amounts of cortisol C^{14} is impaired (Fig 7) (Brown *et al* 1954 Peterson and Wyngaarden 1955 Cameron 1957 Englert *et al* 1957 Tyler 1957) The limiting step seems to be an inability to reduce the unsaturation in ring A since tetrahydro cortisone is cleared at normal rates from the plasma of patients with cirrhosis of the liver (Brown *et al* 1954 Englert *et al* 1957)

In patients with cirrhosis of the liver the 17 hydroxycorticosteroid levels are within the normal range and the miscible pool is nearly normal The reduced urinary excretion of 17 hydroxycorticosteroid and the slow rate of metabolism of large doses of cortisol as well as near tracer amounts of cortisol C^{14} would seem to point to the likeli hood that the adrenal cortex secretes less cortisol in patients with liver damage The mechanisms responsible for these changes are unknown

In the absence of ascites the glucosiduronate metabolites of injected cortisol show a more gradual rise and a lower peak in plasma with maintenance of fairly high levels when compared to the levels in the plasma of normal subjects given cortisol In patients with ascites the glucosiduronate levels tend to be much lower indicating a larger volume of distribution (Englert *et al* 1957)

Recent evidence indicates that in patients with cirrhosis of the liver there is a decreased rate of corticosterone production similar to that of cortisol but in contrast to cortisol the miscible pool and the plasma levels of corticosterone are lower than those in normal subjects In fused corticosterone was metabolized only slightly less rapidly than in normal subjects In all probability the combination of essentially normal metabolism of corticosterone and decreased synthesis of the steroid by the adrenal cortex account for the decreased miscible pool and lowered plasma levels of corticosterone (Peterson 1959)

Effects of Thyroid Function

The plasma 17 hydroxycorticosteroid levels in patients with hyper thyroidism or hypothyroidism are within the range observed for normal subjects although the levels in hypothyroid patients are some what higher than in hyperthyroid patients (Levin and Daughaday 1955 Brown *et al* 1958 Peterson 1958)

Following the injection of cortisol (1 mg per kilogram) into patients with hyperthyroidism unconjugated 17 hydroxycorticosteroids were cleared from the plasma much faster than in normal subjects (Fig 7) The plasma levels of conjugated 17 hydroxycorticosteroids (glucosi

exact significance of this finding in relation to corticosteroid metabolism remains unclear, although the finding of relatively large amounts of unconjugated 17 hydroxycorticosteroid (cortisol) in the urine of newborn infants (Grumbach) may be related to the low transcortin levels (see above)

Effects of Liver Function

Even though evidence is accumulating that extrahepatic tissues may metabolize cortisol existing data point to the liver as the most important organ in the metabolism of cortisol It is of particular importance for conjugation It has been amply demonstrated in patients with liver disease that the clearance of unconjugated 17 hydroxy

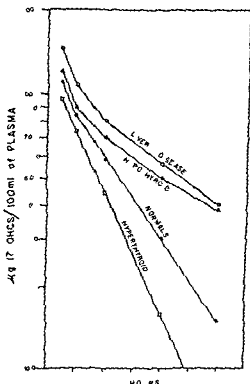


FIGURE 7 The disappearance of 17 OHCS from the plasma of normal subjects patients with liver disease (cirrhosis) subjects with hyperthyroidism or hypothyroidism The half time for the normal group was 100 minutes for the cirrhotic group 240 minutes for the hyperthyroid subjects approximately 80 minutes that for the hypothyroid group could not be determined because equilibrium was not reached in the period studied On the other hand note rapid equilibration in the hyperthyroid subjects All subjects were injected with cortisol (1 mg per kilogram of body weight) in one rapid injection

formation of cortisol In addition factors in the plasma such as protein composition or binding of the steroids may be important

Effects of Renal Disease

In general the clearance of unconjugated 17 hydroxycorticosteroids following the injection of cortisol into patients with renal disease is within normal limits (Englert *et al* 1958) In some patients with renal disease however there may be slightly slower clearance possibly related to hepatic dysfunction which may occasionally accompany severe kidney damage (related to cardiac failure inanition or other factors) On the other hand the conjugated 17 hydroxycorticosteroids in the plasma are maintained at very high levels with some direct relation to the endogenous creatinine clearance The maintenance of high plasma levels of conjugated 17 hydroxycorticosteroids in renal disease is not surprising in view of the fact that over 90 per cent of the conjugated cortisol metabolites are excreted in the urine of subjects with normal renal function

Pregnancy

The unconjugated plasma 17 hydroxycorticosteroids in pregnancy show *a gradual rise with the advance of pregnancy reaching a maximum* sometime in the third trimester (Table IV) Despite this no

TABLE IV Plasma 17 Hydroxycorticosteroid Levels in Nonpregnant and Pregnant Women in the Third Trimester

Authors	Micrograms 17 OHCS 100 ml of plasma	
	Nonpregnant Women	Pregnant Women
Migeon et al (1957)	15 5 ± 6 3	23 3 ± 9 2
Robinson et al (1955)	12 0 ± 1 1	33 0 ± 1 4
Gemzell (1953 1954)	6 6 ± 0 95	25 0
Bayliss et al (1955)	9 5	24 0 ± 1 6
Assali et al. (19 5)	7 0 15 0	34 6 (25 6 47 5)

signs of classic hypercorticism appear The increased levels have been shown to be due not to increased production of cortisol but rather to impaired metabolism of the steroid (Migeon *et al* 1957 Cohen *et al* 1958) It has been demonstrated recently that following the

duronates) achieved very high concentrations in a much shorter period of time than in normal subjects Additionally it was shown that patients with hyperthyroidism conjugated injected tetrahydrocortisone much faster than normal subjects In patients with hypothyroidism the 17 hydroxycorticosteroids were cleared very slowly following the injection of cortisol and the conjugated 17 hydroxycorticosteroids rose slowly and were low in concentration The conjugation of tetrahydro cortisone was nearly normal The response of unconjugated 17 hydroxycorticosteroids in the plasma to an infusion of ACTH revealed higher levels in the hypothyroid group as compared to the hyper thyroid group The opposite was true of the conjugated 17 hydroxy corticosteroids It is interesting to note (see Fig 7) that equilibration between the vascular and extravascular compartments took place rapidly in the hyperthyroid patients (30 minutes compared to 60 minutes in normal subjects) whereas in hypothyroid patients equi libration seemingly never was achieved during the period of study (6 to 8 hours) Treatment of the thyroid condition resulted in return of the metabolism of the 17 hydroxycorticosteroids toward normal

The data indicate that in hyperthyroidism the clearance of cortisol from the plasma and the conjugation of its metabolites occur at more rapid rates than in normal subjects The reverse is true for patients with hypothyroidism Since the latter patients conjugate tetrahydro cortisone normally it would appear that the limiting step in the metabolism of cortisol in these patients as in patients with cirrhosis of the liver is related to reduction of ring A The changes in the plasma levels of the 17 hydroxycorticosteroids during the infusion of ACTH and measurement of urinary 17 hydroxycorticosteroids indicate that adrenocortical secretion is directly related to the clinical state of thyroid function The production of the hyperthyroid state in normal subjects during the administration of triiodothyronine resulted in increased (2 5 times) secretion of corticosterone accompanied by an increased rate of metabolism of infused corticosterone (Peterson 1959)

The exact reasons for the differences in the rate of metabolism of cortisol in the patients with thyroid disease remain to be determined Even though liver function or blood flow may play an important role in the metabolism of cortisol and renal function in the excretion of various metabolites it would seem that other factors may be operating These factors may be related to the influence of thyroxin or its lack on tissue enzymes concerned with various metabolic steps in the trans

berg *et al* 1954a 1956 Bliss *et al* 1954b c Franksson *et al* 1954 Elman *et al* 1955 Steenburg *et al* 1956 Swan *et al* 1957 Viikari and Thomasson 1957 McKay *et al* 1957) In a large measure the elevated levels are due to stimulation of adrenocortical secretion through mechanisms that have not been well defined The amount and kind of stress play an important role in the final effect on the con centration of blood 17 hydroxycorticosteroids Thus it has been shown that following moderate to severe surgical trauma the blood 17 hydroxycorticosteroids may remain elevated for prolonged periods of time Anesthesia may also induce elevated blood 17 hydroxycorticos teroids but in this case the levels are not as high as those seen following surgery and depend greatly on the kind of anesthesia used (ether spinal anesthesia nitrous oxide etc) To some extent the elevated levels of plasma 17 hydroxycorticosteroids induced by surgery are due to impaired metabolism of cortisol (Fig 9) Impaired hepatic function during and following surgery has been demonstrated and this impaired function has been implicated at least in part in the

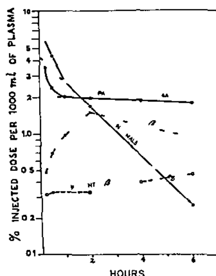


FIGURE 9 Radioactivity in plasma following the injection of cortisol C14 into normal subjects and a patient following gastrectomy for cancer of the stomach (operation lasted 8 hours) The steroid was injected 48 hours following operation The average 17 OHCS in the normal subjects was 12 μg per hundred milliliters The level in the patient was 68 μg per hun dred milliliters It is apparent that substitution of the specific activities at zero time in the formula of Figure 4 reveals a greatly increased miscible pool in the stressed subject This in conjunction with the slower rate of metabolism of the injected cortisol C14 indicates impaired metabolism of the steroid

injection of near tracer amounts of cortisol C^{14} into pregnant women the steroid is metabolized much more slowly than in normal subjects

Another possible explanation for the elevation of the 17 hydroxy corticosteroids in pregnancy has recently been advanced As has been discussed above the preponderant part of plasma cortisol is bound to a special protein transcortin It has been postulated that transcortin bound cortisol is biologically inactive and not available for metabolism Since the levels of transcortin have been shown to be elevated in pregnancy (especially in the last trimester) this finding may explain the elevated 17 hydroxycorticosteroids in pregnancy and the lack of any real signs of hypercorticism (Fig 8) (Slaunwhite and Sandberg 1959)

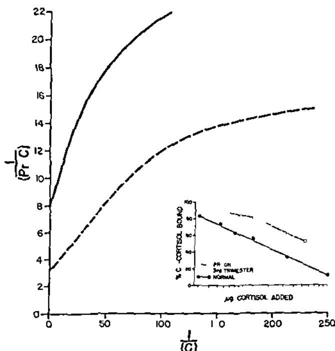


FIGURE 8 The binding of cortisol C^{14} by the plasma of nonpregnant women and pregnant women in the third trimester (Slaunwhite and Sandberg *J Clin Invest* 38 384 1959)

Effects of Stress

Perhaps more work has been done on the effects of stress on adrenocortical function and steroids in blood than in any other condition affecting them Various forms of stress have been shown to be accompanied by elevation of blood 17 hydroxycorticosteroids (Sand

(10 per cent) of cortisol C^{14} has been shown to occur in a dog heart lung preparation These experiments although performed with non human tissues point nevertheless to the fact that the changes observed in the metabolism of cortisol in stressful situations may be related in part to changes in the rate of metabolism of cortisol by extra hepatic tissues Furthermore it has been shown that unusual metabolites of cortisol may appear in the blood of stressed patients (gastrectomized burned terminal cancer patients) pointing to the possibility of abnormal metabolism of cortisol during stress (Weichselbaum *et al* 1957) What part impaired distribution plays in the deviation of 17 hydroxycorticosteroids remains to be determined particularly in regard to the binding of 17 hydroxycorticosteroids by the *plasma proteins and extravascular tissues*

Effects of Medication and Adrenal Insufficiency

It has been demonstrated that prolonged administration of steroid therapy will depress adrenocortical function to the extent that no *demonstrable 17 hydroxycorticosteroids can be found in the plasma of* these patients when therapy is discontinued abruptly (Fig 11) (Sand

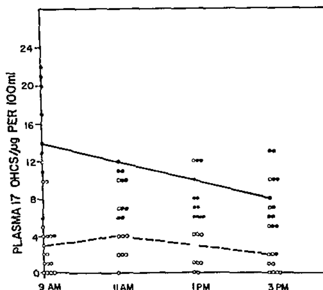


FIGURE 11 Plasma 17 OHCS in patients (open circles) following abrupt cessation of long term steroid therapy the day before the sampling and in untreated subjects (closed circles) The lines represent the means of the plasma 17 OHCS *in the two groups* (Sandberg *et al J Lab and Clin Med* 50 286 1957)

elevation and slower metabolism of the plasma 17 hydroxycorticosteroids (Tyler *et al* 1954)

Children with active rheumatic fever or juvenile rheumatoid arthritis have a markedly decreased rate of metabolism of cortisol (Done and Kelley 1956) During the inactive phase of the disease the metabolism is normal Many other forms of stress cause an elevation of the plasma 17 hydroxycorticosteroids moribund state (Fig 10)

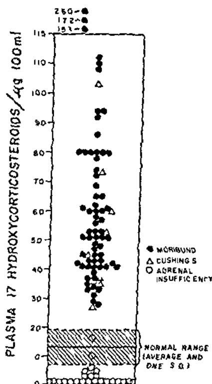


FIGURE 10 Plasma 17 OHCS in dying subjects The levels of normal subjects and patients with adrenal disease are shown for comparison

hypoglycemic shock electroshock burns fever cold etc In all of these cases it has not been established how much of the elevation is due to increased adrenocortical secretion and how much to extra adrenal factors

Recently it has been shown that extrahepatic tissues metabolize cortisol (Berliner and Dougherty 1958 Sweat *et al* 1958 Travis and Sayers 1958) For example mouse fibroblasts *in vitro* have a great capacity to transform cortisol to several of its metabolites (see Fig 3) In addition extensive transformation of cortisol to a series of metabolites by eviscerated rats has been demonstrated Substantial metabolism

may explain why patients on estrogens can tolerate such high levels of cortisol as well as explain the slower metabolism of this steroid in subjects receiving estrogens (Sandberg and Slaunwhite 1959)

The single injection of a bacterial pyrogen (Piromen) has been shown to produce a rise in the plasma 17 hydroxycorticosteroids Studies with cortisol C^{14} in a subject following injection of Piromen revealed a fivefold rise in the miscible pool and a sixfold increase in the rate of turnover of cortisol and demonstrated that this pyrogen increased adrenocortical secretion of cortisol without any significant change in the rate of metabolism of this steroid Using similar methods it was not possible to show any changes in cortisol metabolism following the administration of large therapeutic doses of salicylates (Peterson 1959)

CORTICOSTERONF

Very little is known regarding the effects of extra adrenal factors on the metabolism and concentration of corticosterone in the plasma This has been primarily due to the lack until recently (Bondy and Upton 1957 Peterson 1957a b Ely 1958 Silber *et al* 1958) of an efficient and practical method for the determination of corticosterone levels in the blood even though the presence of this steroid in the plasma has been definitely established Studies with corticosterone C^{14} have shown that this steroid is metabolized much more rapidly than cortisol C^{14} (Fig 12) (Migeon *et al* 1956e) The radioactivity of the glucosiduronate fraction in the plasma reached a maximum within 1 hour The peak was achieved more rapidly and was of greater magnitude than that after cortisol C^{14} injection The miscible pool for corticosterone and its rate of production by the adrenal cortex cannot be evaluated until the actual concentration of this steroid in the blood can be determined All evidence obtained so far suggests that corticosterone is a minor component of adrenocortical secretion in human subjects (less than 1 mg per day)

Studies with tritium labeled corticosterone have shown a half time of this steroid in the plasma of 0 8 to 1 2 hours a pool size of 200 to 400 μg with 0 6 of the pool turned over per hour or 3 6 mg per day (Peterson 1959)

Studies with methods utilizing isotope dilution techniques or considerable purification of plasma extracts reveal that the concentration of corticosterone in the plasma is in the range of 1 to 2 μg per hundred

berg *et al* 1957a Vermeulen 1958) Nevertheless the clearance and metabolism of large doses or near tracer amounts of cortisol C14 are essentially normal in these subjects It has been shown that the metabolism of cortisol C14 in patients with Addison s disease and with no demonstrable plasma 17 hydroxycorticosteroids varies from the normal in a minor respect (Sandberg and Slaunwhite 1958) The only significant difference is related to clearance of the plasma unconjugated and conjugated radioactivity The unconjugated radioactivity is cleared somewhat more rapidly and the conjugated more slowly than they are in a group of non Addisonian subjects Extrapolation of the slower curve of the unconjugated steroids to zero time revealed that the amount of radioactive steroid present in the total plasma of the Addisonian patients at that time was over twice that seen in normal subjects (35 per cent) and that the miscible pool was tremendously reduced a not unexpected result One can speculate that the finding of much larger amounts of radioactivity in the plasma of Addisonian patients may be due to the fact that in the absence of any demonstrable 17 hydroxycorticosteroids the circulating transcortin and other plasma proteins were able to bind a much larger amount of the injected cortisol C14 with the result that less of the steroid was available for the tissues In addition contraction of the plasma volume and some impairment of renal function may also play an important part in the elevation of conjugated steroids and in the higher concentration of both groups of steroids

Observations have also been made in relation to the effects of estrogens testosterone and progesterone on the plasma 17 hydroxycorticosteroids Thus following the oral administration of ethinyl estradiol for a period of several weeks the plasma 17 hydroxycorticosteroids rose significantly (Wallace *et al* 1957) In addition impaired metabolism of cortisol was demonstrated as a result of the administration of estrogen On the other hand the intravenous administration of testosterone in human serum albumin is associated with a marked but transient lowering of the plasma 17 hydroxycorticosteroid levels (Brown and Migeon 1956) No significant changes occurred when progesterone or pregnenolone was given

The levels of plasma corticosterone rise following the administration of estrogens but the rise is not proportionally as great as that of cortisol In this connection it has been recently demonstrated that the transcortin levels are greatly increased by the administration of estrogenic substances These increases in plasma transcortin levels

isotope derivative dilution method the aldosterone concentration in the plasma has been found to be 0 04 to 0 08 μg per hundred milli liters Unfortunately the method requires rather large volumes of plasma (50 to 75 ml) The half time of aldosterone in the plasma of normal subjects has been found to be 0 5 to 0 8 hour (Peterson 1959)

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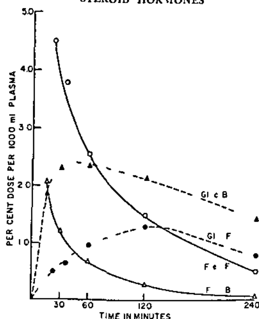


FIGURE 12 The clearance of unconjugated (free) and glucosiduronated radio activity from the plasma of normal subjects following the intravenous administration of cortisol C14 and corticosterone C14 (B) (Migeon *et al* *J Clin Endocrinol and Metabolism* 16 1291 1956)

milliliters of plasma This is below the range that could be accurately measured by any techniques available until recently Even though up to now no reliable method has existed for the accurate measurement of the concentrations of corticosterone in small amounts (30 to 50 ml) of blood several recently developed techniques may prove of value in the determination of the levels of corticosterone and thus aid in the study of the metabolism miscible pool and turnover of this steroid

ALDOSTERONE

Even less is known about the levels of aldosterone in human blood than we know about corticosterone Evidence indicates that the levels are very low (less than 0 1 μg per hundred milliliters) (Ayers *et al* 1957) No good method exists for its direct measurement in the blood

Studies with tritium labeled aldosterone have revealed a much shorter half life in the plasma for this steroid than for cortisol or corticosterone These observations are of interest since preliminary evidence seems to indicate that transcortin does not bind aldosterone to the same extent as it does cortisol or corticosterone Using a double

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CHAPTER XII

Estrogens in Plasma*

Lewis L Engel *and* Clive B Cameron

ESTROGENS ISOLATED FROM TISSUES AND BODY FLUIDS

METHODS OF ASSAY
Biological methods
Chemical methods

NATURE OF CIRCULATING ESTROGENS

FATE OF EXOGENOUS ESTROGEN IN BLOOD

CONCLUSION

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Interest in the secretory activity of the estrogen producing glands has directed attention to the channel by which hormonal agents are transferred from the sites of their formation to their sites of action and their transformation products carried prior to excretion This role of blood as the carrier of both primary secretory products and their metabolites has greatly complicated the study of blood estrogens

The term estrogen should logically apply only to compounds which are active in the production of estrus but it has been extended by common usage to cover substances inducing various uterine changes as well It is convenient to extend this coverage still further to include the steroid metabolites of true estrogens even when the metabolites are completely inactive and it is in this comprehensive sense that the term will be used here

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that this compound and estrone are readily interconvertible by dehy drogenases which are widely distributed Thus a mechanism is present for the reversible modification of biological activity in many tissues (Ryan and Engel 1953)

There is also evidence that tissues other than those endocrine glands cited above can produce estrogens provided that a suitable precursor is presented Thus West *et al* (1956) have shown that estrone and estradiol appear in the urine of ovariectomized adrenalectomized patients with breast cancer following the administration of tes tosterone It has also been reported that the administration of de hydroepiandrosterone (Nathanson *et al* 1952) and 19 nortestosterone (Engel *et al* 1958) leads to increased concentrations of urinary estro gens

Although for many years estrone and estriol were considered to be the principal steroids derived from estradiol in human metabolism (Dorfman and Ungar 1953) recent interest in this field has resulted in the isolation of a number of new metabolic transformation products Three of the four possible 16 17 ketols have been isolated Levitz *et al* (1956) isolated 16 ketoestradiol (IV) from urine after administration of estradiol 16α Hydroxyestrone (V) has been isolated from the urine of pregnant women (Marrian *et al* 1957) as has 16β hydroxyestrone (VI) (Layne and Marrian 1958) 16β *Hydroxyestrone has also been* identified in urine after administration of estradiol (Brown *et al* 1958) Slaunwhite and Sandberg (1956) found 16 ketoestrone (VII) as a *metabolite of administered estrone Epiestriol (VIII) isolated by* Mar rian and Bauld (1955) is the 16 hydroxy epimer of estriol The exist ence of a distinct new class of metabolites was heralded by the isolation of 2 methoxyestrone (IX) as a metabolite of estradiol (Kraychy and Gallagher 1957 Engel *et al* 1957) and as a constituent of pregnancy urine (Loke and Marrian 1958) The estriol analogue 2 methoxyes triol (X) has also been isolated as a metabolite of estradiol in man (Fishman and Gallagher 1958) Yet a further compound 18 hydroxyes trone (XI) has been isolated from pregnancy urine (Loke *et al* 1958) and equilenin (XII) a substance long known to be a constituent of mare pregnancy urine has been identified in human adrenal tumor tissue (Salhanick and Berliner 1957) Thus twelve compounds are now known which must be considered in any discussion of estrogen metabolism and the blood levels of circulating estrogens

ESTROGENS ISOLATED FROM TISSUES AND BODY FLUIDS

It seems worthwhile to outline briefly the present state of our knowledge of the mechanisms and sites for estrogen production and the nature of the known transformation products which have been isolated limiting the discussion to those compounds which have been isolated from human tissues or body fluids except where information from other species seems directly relevant (see Fig 1)

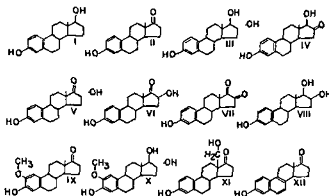


FIGURE 1 Structure of estrogens isolated from tissues and body fluids

A definitive demonstration of the formation of a hormone by a particular tissue consists in showing a concentration difference between the afferent and efferent blood vessels Because amounts of estrogens produced by their principal sources in the nonpregnant individual are exceedingly small no such proof has yet been adduced for the estrogens However the isolation studies performed by the pioneers in endocrinology disclosed the presence in sow follicular fluid stallion testes beef adrenal glands and human placentae of estradiol 17β (I) (hereafter called estradiol) estrone (II) and in the case of placenta estriol (III) as well (Dorfman and Ungar 1953) Although the isolation of these substances from tissues by no means constitutes proof of their formation there recent biochemical data demonstrate the presence in these endocrine tissues of biochemical pathways for the formation of estrone and/or estradiol from Δ^4 androstene 3 17 dione 19 hydroxy Δ^4androstene 3 17 dione and testosterone (Engel 1957 Ryan 1958 Baggett *et al* 1959) It is generally believed that estradiol is the primary secretory product in the human being and it has been shown

may exist under usual conditions is suggested by the early findings that some mapping out of the relatively marked changes in blood estrogen levels in pregnancy and the menstrual cycle is possible by the use of bioassay but such an assumption is too sweeping to be gen erally acceptable without further evidence If the assumption is true results obtained by *bioassay of mixtures of unknown estrogens in blood* or blood fractions can be compared with the results obtained on the same type of sample under identical experimental conditions but cannot be related to absolute values in terms of for example inter national standard estrone or usefully compared with results obtained by different methods or even the same method in different laboratories Nor can the application of bioassay to partially purified preparations of single components be considered reliable because of the great dif ferences in estrogenic potency among the several estrogens Trace contamination of estriol with estradiol for example will completely invalidate quantitative assay of an estriol fraction by many bio logical methods

These and other limitations make it unlikely that bioassay proce dures despite their appealing sensitivity can take us very far in defining either the nature of the circulating estrogens or the concen trations in which they occur The development of chemical methods of sufficient sensitivity has marked the beginning of a more definitive phase of blood estrogen studies and it may be useful at this point to consider briefly chemical techniques at present available and their shortcomings

Chemical Methods

Sensitive and specific colorimetric and photofluorometric methods now exist for the determination of purified estrogens A color reaction described by Kober (1931) was first applied to urinary extracts for the estimation of estrone and estriol by Cohen and Marrian (1934) Brown (1952) subsequently reinvestigated the Kober reaction introducing several modifications which greatly increase its reliability Quantita tive photofluorometric methods for the estimation of estrogens were introduced (Bates and Cohen 1947 Jailer 1947 Finkelstein *et al* 1947) Subsequent study of these procedures has greatly improved their specificity and sensitivity (Umberger and Curtis 1949 Bates and Cohen 1950 Aitken and Preedy 1953 Slaunwhite *et al* 1953 Goldzieher *et al* 1954) Both colorimetric and photofluorometric methods give reliable quantitative information however only when

METHODS OF ASSAY

Biological Methods

Early in the history of this field it was recognized that estrogen metabolites do not occur in the urine in the free form but rather as conjugates subsequently identified as either glucosiduronates (Cohen and Marrian 1936) or sulfates (Schachter and Marrian 1938) Enzyme systems responsible for these conjugation reactions have been found in relatively high concentration in liver tissue These systems include the kinases which transfer the glucuronic acid moiety from uridine di phosphate glucuronic acid to steroid (Mills *et al* 1958) and those which catalyze the transfer of sulfuric acid from phosphoadenosine phosphosulfate to steroid (Lipmann 1958) Conjugated forms of estro gen metabolites must also be sought in blood Thus the simple problem originally presented that of measuring the level of estrogen in blood is seen to acquire more complex ramifications First it is clear that unless one is to deal exclusively with biologically active steroids and thus eliminate from consideration significant metabolites which no longer possess biological activity some chemical method is required for the measurement of total estrogens in blood The diversity of chemical compounds represented among the metabolites together with their low concentration places such a severe limitation on the type of chemical method that can be used that to date no method is avail able to measure all of them This matter is further confused when it is realized that no native endogenous estrogen has yet been isolated from human blood and characterized by criteria satisfactory to an organic chemist

Ignorance of the nature and extent of conjugation and protein binding has greatly handicapped the methodology of extraction and purification and the interpretation of bioassay data In the latter connection Emmens (1950) has convincingly demonstrated that only with carefully designed and analyzed experiments is it possible to ex press the activity of a single highly purified estrogen in terms of international standard estrone and therefore with other highly puri fied estrogens The use of bioassay to measure unknown mixtures is fraught with hazards Data on total estrogenic activity in different bloods or blood fractions are comparable only if all estrogenic com ponents whether free conjugated or protein bound are present in the same proportions and any interfering substances are also present in constant proportions That this state of relatively constant proportions

studies concerned with the nature of unknown endogenous estrogens and other studies on the fate of known exogenous estrogens

NATURE OF CIRCULATING ESTROGENS

Estrogenic activity was detected in blood extracts by Loewe (1925) in Estonia and Frank *et al* (1925) in the United States using the sensitive bioassay methods made available by the pioneering work of Allen and Doisy (1923) Frank and Goldberger (1926) reported that the concentration of estrogen in human female blood varies throughout the menstrual cycle increasing at the tenth to fifteenth day and again near the first day of menstruation Muhlbock (1939) noted that from one third to one half the estrogenic activity in blood was extractable only after treatment with hydrochloric acid and postulated a conjugated form of estrogen such as had previously been shown to occur in urine Albrieux (1941) reported estrogenic activity in both red cells and plasma A steady increase in blood estrogen concentration during pregnancy with an abrupt fall at labor was recognized (Goldberger and Frank 1942) More elaborate studies of human pregnancy serum by Rakoff *et al* (1943) provided further evidence that a proportion of the estrogens of human pregnancy serum is conjugated They suggested also that estrogens are intimately bound to protein a concept which had been put forward previously by Brunelli (1935) and Haussler (1936) and which is still a subject of investigation and controversy (see Antoniades Chapter XIV)

Attempts to define the nature of circulating estrogens have met with such difficulties that up to the present no estrogen in normal or pregnancy blood appears to have been isolated and characterized unequivocally Using a method compounded of solvent partition hot acid hydrolysis alkaline hydrolysis and photofluorimetry Diczfalusy (1953) studied the estrogen content of blood taken immediately post partum and was able partially to characterize estriol and adduce suggestive evidence for the presence of estrone In each of the blood samples the concentration of estriol was five to ten times that of estrone with average figures of 122 μg and 17 μg per kilogram of blood respectively Preedy and Aitken (1957) studied plasma estrogen levels in late pregnancy in the normal menstruating female and in the male Using acid hydrolysis partition chromatography and photofluorometry they found values in late pregnancy blood ranging from approximately 2 to 10 μg for estrone less than 3 μg for estradiol and

applied to extracts containing single estrogens free from interfering contaminants (Bauld and Greenway 1957) Trace contamination of one estrogen with another is in general less important in chemical assay than it is in bioassay as the Kober color and fluorescent characteristics of the main estrogens are more uniform than their biological activity However adequate purification of estrogen extracts from biological fluids is obviously a necessary preliminary to the isolation characterization and quantitation of the estrogens under study Classic procedures for the extraction and purification of estrogens utilize their general property common to all steroids of high solubility in organic solvents and the special properties conferred by a phenolic ring A More recently countercurrent distribution and partition chromatographic procedures consisting essentially of fractionation based on distribution coefficients have proved invaluable for the separation of closely related estrogens Countercurrent distribution first put on a practical footing by Craig (1944) has the virtue that it can achieve purification separation and quantitation of individual components in a mixture It can be used for partial characterization as the distribution coefficient of a compound in an exactly prepared solvent system and is as exact a physical property as the melting point refractive index solubility or absorption spectrum (see Weisiger 1954 for an excellent account) Engel *et al* (1950) applied the countercurrent distribution method to the separation of urinary estrogens and Diczfalusy (1953) has used it for the purification and partial characterization of estrogens from blood While various stages are established for the concentration and assay of certain of the estrogens in blood no complete method has been developed and subjected to critical evaluation in terms of sensitivity specificity accuracy and precision This goal has been attained for urinary estrone estradiol and estriol in the two methods developed in the Edinburgh laboratories (Brown 1955 Bauld 1956 Marrian 1956) It is premature at this time to consider total blood estrogen measurements of sufficient quantitative significance for useful clinical application For a clinical method for blood estrogens to be justifiable it must measure at least part of the total estrogens present and if part only is measured this part must be representative of the total Furthermore if unrelated substances are also measured or interfere in the measurement they must always be present in the same concentrations These conditions cannot yet be fulfilled

Because of uncertainty regarding the nature of estrogen entering the circulation we have considered it desirable to distinguish between

obtained suggestive evidence for the presence of estriol in such pre cipitates However Bischoff and Stauffer (1957) have been unable to detect estrogenic activity in ethanol precipitated plasma proteins The conclusions drawn about protein binding of circulating estrogen from precipitation data are valid if it can be assumed that precipitation *per se* does not result in dissociation or association Accurate stoichiometry is clearly impracticable at present but such data as exist suggest that the estrogen protein ratio is fairly constant in plasma protein pre cipitated in a variety of ways (Szego and Roberts 1946)

Purified human plasma protein fractions prepared by Cohn frac tionation (Cohn *et al* 1946) have been tested for estrogenic activity with conflicting results Roberts and Szego (1946) using a rat uterine weight technique highly sensitive to estriol found practically all the estrogenic activity selectively concentrated in Fraction III 0 (β lipo protein) from which it was not removed by cold ether but was re moved by dialysis under simulated physiological conditions Sandberg *et al* (1957) using a different bioassay technique found estrogenic activity in Fractions III and IV 1 (α-lipoprotein) but none in Fraction III 0 Bischoff *et al* (1954) also failed to find estrogenic activity in Fraction III 0

There is a greater measure of agreement on the dialysis studies of blood or plasma Szego and Roberts (1946) reported that estrogenic activity in pregnancy blood dialyzed into distilled water almost quan titatively within 72 hours They postulate an estrogen β lipoprotein complex of such a nature that dialysis results in its progressive disso ciation perhaps by preserving the ratio between dissociated and protein bound estrogen within the dialysis membrane Bischoff and Stauffer (1957) dialyzed pooled serum from subjects in the third trimes ter of pregnancy against human serum albumin solutions found partial equilibration of endogenous estrogen between the two components and conclude that albumin is an important factor in the orientation of circulating estrogens Estrogenic activity cannot readily be ultra filtered from blood plasma (Roberts and Szego 1946) Further data on protein binding derive from studies of added or exogenous estro gens of known structure and these will be discussed below as they have their own potential fallacies which are distinct from those inherent in studies of endogenous blood estrogens Better understanding of the latter must await more definite techniques which in turn are dependent on a clearer knowledge of the estrogens involved

4 to 18 μg for estriol per hundred milliliters of plasma Assuming for the purposes of comparison that plasma contains the main bulk of the circulating estrogen these figures compare reasonably with Diczfalusy s findings But whereas Diczfalusy found a fairly constant relationship between estrone and estriol levels no such constancy is suggested from the data of Preedy and Aitken Much lower levels were found in normal menstruating females and levels in normal males were unmeasurable (Preedy and Aitken 1957) It should be re emphasized that the reliability characteristics of the methods used have yet to be determined

Evidence that a part of circulating estrogen is present in a conjugated form is at the present only indirect resting on the demonstration that more estrogenic activity can be extracted with organic solvents from blood after the latter has been treated with hot acid No naturally occurring estrogen conjugate has been isolated from blood The data of Muhlbock (1939) Rakoff *et al* (1943) and Szego and Roberts (1946) suggest that about one third of normally circulating estrogen is in the conjugated form hydrolyzable by hot acid but this proportion of conjugated to unconjugated estrogen may be revised when the question is studied with techniques more accurate and specific than the bio assay procedures used

Observations based on chemical assay are scanty Diczfalusy (1953) concluded from his findings in pregnancy blood that circulating estrone is conjugated and estriol both conjugated and free Calculations from these data suggest that conjugated estrogens account for from one half to four fifths of the total circulating estrogens averaging about two thirds Further important clues as to the extent of conjugation of blood estrogens have been provided from studies of exogenous estrogen in blood (see p 408)

The relationship of endogenous circulating estrogen to the plasma proteins has been studied by a number of workers They have had to contend with many difficulties so it is not surprising that their findings are varied and difficult to interpret The main difficulty is evident enough — lack of any precise knowledge concerning the molecular structure or state of conjugation of the estrogens under study This has magnified problems in methodology and made many results ambiguous Evidence for protein binding has been inferred from a variety of experiments Early workers showed that estrogenic activity is demonstrable in washed plasma protein precipitates (Haussler 1936 Rakoff *et al* 1943 Szego and Roberts 1947) and Diczfalusy (1953)

cluded the definite isolation of any metabolite of administered estrogen in *in vivo* experiments

In vivo studies with administered estrogen have also been directed to an understanding of the nature and extent of conjugation in the circulating estrogen Sandberg and Slaunwhite (1957) reported that 15 minutes after the injection of estrone C^{14} or estradiol C^{14} a fraction of the radioactivity in plasma can be extracted only after β glucuronidase hydrolysis This fraction is somewhat higher when estradiol is injected rather than estrone This level of circulating glucosiduronate persists at about 3 per cent of the injected dose (approximately 2 per cent for estrone) for about 2 hours and thereafter slowly declines There is also evidence for the conversion of the administered estrogens to small amounts of other conjugates perhaps sulfates cleaved by extraction with ether at pH 1 Wotiz *et al* (1958) confirmed the presence of conjugated estrogens in rat plasma fractions — conjugated that is by the indirect criterion of greater organic solvent solubility after various forms of hydrolysis No conjugated estrogen in blood has been isolated or characterized

Sandberg *et al* (1957) have studied the relationship of estrogen to the plasma proteins by examining the distribution of radioactivity in the blood of subjects injected with estrone C^{14} They concluded from their results that a small but definite proportion of the total radioactivity in the blood was associated with the proteins in a form not extractable with acetone This radioactivity was found preferentially concentrated in Fraction V (predominantly albumin) when the plasma proteins were fractionated according to methods 6 and 9 of Cohn *et al* (1946)

Results of *in vitro* studies of the protein binding of exogenous estrogens have not always paralleled the findings from *in vivo* experiments and it is clear that *in vitro* experiments involve many variables at present inadequately controlled That more than physicochemical factors may be involved is suggested by Szego and Roberts (1953) who have reviewed the evidence for a specific role of the liver in the formation of estrogen protein complexes It seems certain that a more meaningful understanding of the extent and mechanisms of protein binding must await the better isolation and characterization of both the estrogen and protein components concerned

Factors similar to those involved in plasma protein binding probably determine the distribution of estrogen between red cells and plasma Estrogenic activity was demonstrated in both red cells and plasma (Albrieux 1941) and Sandberg *et al* (1957) found significant radio

FATE OF EXOGENOUS ESTROGEN IN BLOOD

There have been several reports on the distribution and metabolism of exogenous estrogens under *in vivo* and *in vitro* conditions Underlying most of these experiments is the assumption that by infusing or adding small amounts of free estrone or estradiol one is simulating normal endogenous estrogen secretion However it should be recalled that the nature of the primary hormone whether free or conjugated estrone or estradiol is still uncertain The amount of estrogen that can be infused *in vivo* must be small if conclusions as to normal physiological behavior are to be drawn Flooding the organism with a considerable excess of hormone may promote pathways of distribution and metabolism not normally operating Carbon 14 estrogens have proved invaluable in this regard as metabolites can be followed in minute concentration It is anticipated that tritium labeled estrogens will be even more useful in metabolic studies because of the very high specific activities that can be attained

When estradiol C^{14} is given intravenously to human subjects the concentration of radioactivity extractable from plasma without prior hydrolysis rapidly falls (Sandberg and Slaunwhite 1957) Only about 5 per cent remains 15 minutes after infusion and less than 2 per cent after 1 hour this free steroid fraction is negligible 8 hours after injection Within a few minutes the rest of the administered estrogen has left the peripheral circulation partially equilibrated with the extravascular space entered the biliary excretory pathway and begun to be excreted in the urine Metabolites returning from extravascular sites re-enter the circulation (Sandberg and Slaunwhite 1957)

Ryan and Engel (1956) attempted to identify some transformation products of an administered estrogen conjugate Human serum obtained after a massive infusion of potassium estrone sulfate (900 mg over 18 hours) after dialysis organic solvent extraction and treatment with acid was shown to contain fluorogenic phenols with the partition characteristics of estrone estradiol and estriol Lack of material precluded definitive identification of estradiol and estriol Wotiz *et al* (1958) studied rat plasma after a 5 minute infusion of 100 μg of esterone C^{14} (in oil) and provided evidence for the presence in hydrolyzed extracts of labeled estradiol and a more polar metabolite thought by them to be estriol However technical difficulties have so far pre

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activity in both red cells and plasma after intravenous injection of estrone C^{14} and estradiol C^{14} Attempts to define this partition more closely must also attend specific knowledge of the chemical nature of the blood estrogens themselves

Speculation as to whether the blood itself is responsible for the metabolic conversions of the estrogen it carries have been tested by experiment but remain unresolved because of conflicting evidence or ambiguities inherent in the experimental procedures used Fee *et al* (1929) and Israel *et al* (1937) found no loss of estrogenic activity from blood on standing but the extraction techniques used are now known to have been incomplete Werthessen *et al* (1948) incubated estrone with human blood and concluded from their results that there was con version of estrone to an alcoholic ketonic derivative Levy (1954) was unable to confirm this finding recovering added estrone virtually quantitatively Bischoff and his collaborators (Bischoff *et al* 1952 Gray and Bischoff 1955) have incubated red cells with estrone and obtained evidence for enzymatic conversion of estrone to estradiol No products have however been characterized unequivocally

CONCLUSION

It can be seen from the foregoing that because of the fragmentary nature of our knowledge of blood estrogens only a rough qualitative outline can be discerned There is a rapid turnover of secreted estrogens which circulate partly conjugated and partly free Plasma al bumin and perhaps other plasma protein fractions appears to be im portant for binding estrogens Either red cells or plasma or both transport estrogens and may contain enzymes for estrone estradiol inter conversion and other metabolic conversions The quantitative aspects of these many factors remain obscure

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CHAPTER XIII

Progesterone

William H Pearlman

THE ESTIMATION OF PROGESTIN IN BLOOD
- Rabbit progestational proliferation tests
- *McGinty rabbit intrauterine test*
- Hooker Forbes mouse intrauterine test
  - Sensitivity and precision of the Hooker Forbes test
  - Specificity of the Hooker Forbes test

PROGESTIN (HOOKER–FORBES) LEVELS IN BLOOD
- Pregnancy and the puerperium
  - Human beings monkey
  - Rabbit ewe mouse
- Menstrual cycle (human beings monkey)
- Estrous cycle (rabbit ewe)
- Ovulatory cycle (fowl)
- Male subjects the role of the adrenal cortex and testis as sources of progestin
  - *Human beings*
  - Bull ram rabbit hamster squirrel rat
  - Turtle snake frog fowl

PHYSICOCHEMICAL METHODS OF ESTIMATING PROGESTERONE IN BLOOD
- Extraction of blood
- Fractionation of blood extracts countercurrent distribution partition Column chromatography paper chromatography
- Estimation of progesterone
  - Ultraviolet spectroscopy
  - Infrared spectroscopy
  - Polarography
  - Fluorometry
  - Qualitative tests for progesterone

PROGESTERONE BLOOD LEVELS (PHYSICOCHEMICAL ESTIMATIONS)
- Pregnancy
  - Human beings
  - Cow ewe mare
- *Menstrual cycle (human beings)*
- Estrous cycle (ewe)

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Methods for estimating progesterone or progesterone like substances in blood may be classified as (1) biological and (2) physicochemical The latter are as a general rule much less time consuming more definitive and more precise On the other hand bioassay data are of greater physiological interest and are most illuminating when comparison is made with *physicochemical estimates There appear to be* serious discrepancies between the two sets of data which indicate that our knowledge is incomplete with respect to the nature and precise amounts of circulating gestagens (progestationally active substances) and to the factors present in blood which may enhance or diminish their biological action

At present quantitative physicochemical estimation of progesterone in peripheral blood is limited to studies in advanced pregnancy the amounts of progesterone during the menstrual cycle and during early pregnancy are too small to be measured unless very large volumes of blood are obtained A need for the development of more sensitive chemical methods for the estimation of progesterone in blood is apparent An intrauterine bioassay procedure (Hooker Forbes) meets the requirement of high sensitivity but has certain limitations An indirect approach to the study of progesterone and its metabolites in circulating blood has recently been made in metabolism experiments entailing the intravenous administration of trace amounts of radio active progesterone

An integrated view of the hormonal status of the individual under various physiological conditions has emerged in a few studies which attempt to correlate the rate of endogenous elaboration of progesterone the circulating level of progesterone and the rate of urinary excretion of pregnanediol Factors other than the rate of endogenous hormone production (p 440) are also *important in influencing the* level of circulating progesterone

Emphasis in this review is on studies in man as the title of this volume implies It would be regrettable however to omit pertinent studies on animals as these have an important bearing on human physiology but it must be kept in mind that notable differences exist *among the species with respect to (1) the role of various endocrine* organs especially the placenta as contributors to the circulating pool of progesterone and (2) the metabolism of progesterone Also the relative progestational activity of various compounds is dependent on the species employed as a test subject and indeed on the nature of the test itself In the following presentation the term progestin refers

FACTORS INFLUENCING THE LEVEL OF CIRCULATING PROGESTERONE
Rate of endogenous progesterone production
Rate of disappearance of progesterone from the blood stream
Rate of turnover metabolism and the rate of turnover

THE TRANSPORT OF PROGESTERONE IN BLOOD

PROGESTERONE METABOLITES IN PERIPHERAL BLOOD

PROGESTERONE METABOLISM SOME MAJOR ASPECTS

BIBLIOGRAPHY

It is remarkable that despite the prodigious quantities of progesterone elaborated daily in advanced human pregnancy the concentration of the hormone in peripheral blood remains very low During the menstrual cycle the circulating hormone level is even lower which makes quantitative estimation by chemical methods very difficult indeed Our knowledge of the precise amount of progesterone in human plasma and of the nature of the metabolites accompanying it under normal physiological and pathological conditions is on the whole very limited There is however considerably more information regarding the nature and the amounts of the progesterone metabolites excreted in the urine under various conditions Thus the chemical estimation of urinary pregnanediol has provided a very useful and fairly reliable index of the level of endogenous production of progesterone But the rate of urinary excretion of pregnanediol — progesterone is present in barely detectable amounts in pregnancy urine — may be considered at best a reflection of the hormonal status of the individual especially since the degree of metabolic conversion of progesterone to urinary pregnanediol varies appreciably from one individual to another also progesterone may not be in all circumstances the sole or major source of pregnanediol Of greater significance is the concentration of the hormone in peripheral blood as this may be a most critical factor in determining the degree of response elicited in the target organs of hormone action More pertinent is the hormone concentration in the blood bathing the target tissues which may or may not be substantially the same as that in peripheral venous blood

(1941) adapted the McGinty test to the quantitative estimation of progestin in blood and tissues In the modified procedure an entire horn of the uterus of the rabbit was injected with 0 2 to 0 3 ml of the hormone in oil and ligated only at the cervix The endometrial response was graded on the McPhail scale from (+) to (+++) corresponding respectively to doses of 0 25 and 1 00 μg of progesterone For the estimation of progestin in human serum about 40 ml of serum was required the serum was extracted with ether and estrogen removed from the ether extract by partitioning with petroleum ether aqueous ethanol before injection The progestin content of peripheral venous blood was thus found (Hoffmann and Lam 1942) to rise progressively with advancing pregnancy to a maximum of about 0 018 μg equivalents of progesterone per milliliter of serum the peak occurring in the last few months of human pregnancy following parturition progestin dropped sharply to prepregnancy levels Circulating progestin levels during the course of the normal menstrual cycle in women were similarly studied (Hoffman and Lam 1948) Prior to the fourteenth day of the cycle serum progestin was not detectable on the eighteenth to twenty fourth day progestin appeared in a concentration of 0 003 to 0 004 μg equivalents of progesterone per milliliter of serum subsequently the progestin level declined It is noteworthy that the patterns of circulating progestin observed in these studies are very similar to established patterns of urinary pregnanediol levels under the same physiological conditions i e during the human menstrual cycle and pregnancy (Venning and Browne 1937 Venning 1948) It is of pertinent interest to mention that from the latter studies (Venning 1948) there is no indication that a marked fall in progesterone elaboration occurs several days prior to the onset of labor on the other hand it appears that as soon as the placenta begins to separate there is a rapid drop in urinary pregnanediol output

It is disturbing to note however that the progestin content of blood reported by Hoffmann and Lam (1942) is considerably lower than the progesterone content subsequently estimated by physicochemical methods thus Zander (1955) found an average of 0 142 μg (range 0 039 to 0 268 μg) of progesterone per milliliter of plasma in advanced human pregnancy a value eight times greater than the progestin value (in terms of microgram equivalents of progesterone) This discrepancy may possibly be accounted for by an incomplete extraction of progestin from the serum

to progestationally active substances and the test employed will be indicated

THE ESTIMATION OF PROGESTIN IN BLOOD

Rabbit Progestational Proliferation Tests

The classic techniques (Corner and Allen 1929 Clauberg 1930 and McPhail 1934) for the assay of progestational activity require relatively large amounts of hormone (for a review of progestin assay methods see Emmens 1950) A unit dose of 0 4 to 1 0 mg of pro gesterone subcutaneously or intramuscularly administered is required to elicit a full scale progestational proliferation of the uterine endo metrium in the rabbit the presence of estrogen in extracts to be assayed *may vitiate the test Such procedures are too insensitive to* detect progesterone in peripheral blood even during pregnancy Thus Clauberg *et al* (1933) employing his assay method could not detect progestin either in extracts of 335 ml of blood drawn from women during various stages of the menstrual cycle or in extracts of 300 ml of blood from pregnant women Bloch (1936) employing the Corner Allen test failed to detect any progestin in 500 ml of human preg nancy blood and less than one rabbit unit of progestational activity in 8 to 12 liters of sow s blood From these data one may estimate less than 0 1 μg equivalent of progesterone per milliliter of pregnancy blood

McGinty Rabbit Intrauterine Test

A considerably more sensitive method of bioassay is based on the intrauterine application of progesterone Thus McGinty *et al* (1939) demonstrated that progesterone in single doses of 0 5 to 5 μg intro duced into isolated loops of the uterus of immature rabbits previously treated with estrogen produces progestational proliferation equivalent to that brought about by a total of 0 5 mg given intramuscularly in five daily divided doses the histological results were expressed on the McPhail scale As there was considerable variation in response at the same dose level the authors pointed out that further study would be required before this assay procedure could be regarded as quanti tative Haskins (1941) applied the McGinty test to the estimation of circulating progestin in human pregnancy and found less than 0 13 μg equivalents of progesterone per milliliter of serum a positive but minimal reaction was obtained in the assay series Hoffmann and Lám

degrees of luteal activity (McPhail scale) when given systemically to rabbits failed to evoke in the Hooker Forbes test a nuclear response identical with that produced by progesterone (0 0002 μg) even when very large amounts were injected These were ethynyl testosterone (less than 0 006 μg) desoxycorticosterone acetate (6 3 μg) desoxy corticosterone (6 3 μg) methyltestosterone (15 μg) Δ^5 pregnenolone (5 7 μg) and testosterone (0 48 μg) the dose level is given in paren theses Estrone (0 6 μg) estradiol (0 00075 μg) various progesterone reduction products and C_{19} androgens and cholesterol also failed to evoke the response In further tests carried out by Forbes (1954) cortisone acetate (4 μg) and 17 hydroxycorticosterone (0 4 μg) likewise failed to elicit a response A positive response however was given by Δ^{11} progesterone (6 1 μg) and by an unidentified $\alpha\beta$ unsaturated ketone Compound X *(0 008 μg) which had been isolated from cow* pregnancy bile by Pearlman and Cerceo (1948) the latter compound evoked the response in a dose forty times that required for progester one

From the foregoing data it would appear that the Hooker Forbes intrauterine test is highly specific for progesterone However more recent studies by Zander *et al* (1957 1958) revealed that Δ^4 pregnen 20β ol 3 one is at least twice as active as progesterone in the Hooker Forbes test only 0 0001 μg being required to elicit a positive response its stereoisomer Δ^4 pregnen 20α ol 3-one is less active 0 0005 μg being required to produce a response Both epimers are also active in the Clauberg test the 20α ol compound is about ½ to ⅓ and the 20β ol about ⅕ to $\frac{1}{10}$ as active as progesterone Further evidence that the *Hooker Forbes test is not specific for progesterone was obtained by* Zarrow *et al* (1957) Most astonishing was their finding that 17α hydroxyprogesterone is sixty times as active as progesterone in the mouse intrauterine test particularly since this compound is weakly active by the systemic route of administration in the rabbit or human being (Salhanick *et al* 1957) however the 17α acyl derivatives for example the caproate are potent long acting progestational com pounds Other compounds (Zarrow *et al* 1957) found to have considerable activity (Fig 1) in the Hooker Forbes test are given *below the ratio of their activity* to that of progesterone is indicated in parentheses 11 dehydroprogesterone (1 0) 19 nor progesterone (0 3) and 17β hydroxyprogesterone acetate (0 3)

An important consideration in estimating progestin in the Hooker Forbes test is the strain of mice employed in the assay procedure

Hooker Forbes Mouse Intrauterine Test

Sensitivity and Precision of the Hooker Forbes Test The mouse intrauterine procedure devised by Hooker and Forbes (1947 1949a) is considerably more sensitive than the McGinty test and is quantitative The technique requires the injection of 0 0006 to 0 0008 ml of a solution (sesame oil) of the material to be tested for example an ether and acetone soluble extract of plasma directly into a segment of the uterus of an ovariectomized mouse An aqueous solution for example plasma diluted with 0 9 per cent saline may also be injected the sensitivity of the test remaining the same (Hooker and Forbes 1949a) As little as 0 0002 μg of progesterone can be detected in 0 0006 ml of test fluid (at a concentration of 0 33 μg per milliliter) The response to progesterone consists of the transformation of some of the endometrial stromal nuclei which in the castrated mouse are shrunken dense and fusiform into plump slightly elongated oval nuclei with conspicuous nucleoli and fine evenly dispersed chromatin particles The unusually high sensitivity of this test readily permits estimation of progestin in peripheral blood Progestin is expressed in terms of progesterone equivalents Forbes (1955) defines one progesterone equivalent as equal to the activity of 1 μg of progesterone in the Hooker Forbes test in the absence of estrogen and other interfering factors

Specificity of the Hooker Forbes Test In the Hooker Forbes test the identity of the compounds in blood comprising progestin is uncertain (Forbes and Hooker 1957) Extensive data have been obtained by Hooker and Forbes and by Zarrow and his associates on progestin levels in blood with the aid of this test as discussed below the values are considerably higher than those obtained by physicochemical methods Some reasons for this disparity may become apparent in the following discussion of the specificity of the Hooker Forbes test The chemical identity of blood progestin may perhaps be ultimately established by a systematic study of the distribution of progestin in plasma extracts following paper chromatography countercurrent distribution or partition column chromatography

Hooker and Forbes (1947) stated that estrone estradiol 17β desoxy corticosterone acetate and testosterone fail to elicit the reaction The specificity of the test was further examined by the authors (Hooker and Forbes 1949b) and it is curious that the following products which were found by Selye and Masson (1943) to exhibit varying

is wanting Forbes (1955) has cautioned that in view of the report by Ober *et al* (1954) that the sensitivity of the Hooker Forbes assay may be increased up to fivefold in the presence of progesterone estradiol mixtures in the ratio of 500 1 it is possible that the endog enous estrogens in peripheral blood may disturb the accuracy of the bioassay

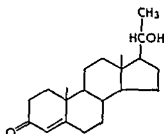
In view of the report by Hisaw and Velardo (1951) that a number of *steroids such as pregnanediol testosterone desoxycorticosterone* acetate and cortisone inhibit progesterone in the development and *maintenance of the decidual reaction Zarrow and Neher (1953) tested* pregnanediol and testosterone propionate in the Hooker Forbes reac tion as much as 500 parts of either of these two steroids failed to block the action of 1 part of progesterone

PROGESTIN* (HOOKER-FORBES) LEVELS IN BLOOD

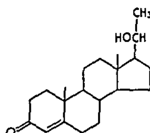
Pregnancy and the Puerperium

Human Beings Monkey Hooker and Forbes (1949a) estimated progestin in peripheral blood (hematocrit 35 7) obtained from a woman in the eighth week of pregnancy at 4 0 μg per milliliter of raw (unextracted) whole blood 5 3 μg per milliliter of raw plasma 5 3 to 5 5 μg per milliliter of plasma in the ether or acetone extracts and 0 2 μg per milliliter of plasma in the acid hydrolyzate of the acetone precipitate of plasma The acetone precipitate contains bound progestin the acetone-ether soluble extract contains free progestin Assay of raw plasma or blood was made by dilution with 0 9 per cent saline and injection of this test solution The ether or acetone extracts were dissolved in sesame oil for injection Acetone extraction was made by slowly adding plasma with stirring to ap proximately 10 volumes of acetone at 3 C to precipitate the proteins From these data it appears that progestin is transported entirely in the plasma and is primarily free rather than bound to protein How ever under certain experimental conditions the ratio of bound to free progestin and also the absolute level of bound progestin can be increased Forbes and Hooker (1949) showed that in ovariectomized mice with subcutaneous and intrasplenic implants of progesterone the concentration of bound progestin attained levels as high as 2 to 3 μg

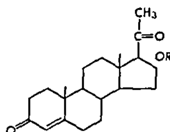
Progestin is expressed throughout as microgram equivalents of progesterone it was determined by the Hooker Forbes test unless otherwise indicated



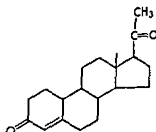
Δ^4 Pregnen 20α ol 3 one



Δ^4 Pregnen 20β ol 3 one



17α Hydroxyprogesterone (R = H)
17α Caproylprogesterone (R = caproyl)



19 nor Progesterone

FIGURE 1 Some potent progestationally active compounds other than progesterone

Zarrow and co workers employ a Rockland Swiss strain whereas Hooker and Forbes employ the CHI strain of mice in their studies Progesterone is equally active in either strain of mice 17α hydroxy progesterone is exceedingly potent in Rockland Swiss mice as pre viously mentioned but devoid of activity in the CHI strain (cited as unpublished data by Zander *et al* 1958)

A factor which may seriously interfere in the Hooker Forbes test is the presence of estrogens in the extract to be assayed this is an addi tional reason for fractionating such extracts before injecting Zarrow and Neher (1953) found that estradiol to progesterone in a ratio of 1 20 by weight inhibited the response in the Hooker Forbes test for estrone to progesterone the inhibiting ratio was 1 1 Studies with estriol failed to indicate any antagonism to progesterone even at a ratio of estriol to progesterone of 60 1 Salhanick *et al* (1951) found a block at a ratio of estradiol to progesterone of 1 200 Zarrow and Neher (1953) believe that the amounts of estrogen present in preg nancy blood are insufficient to interfere in the test However our knowledge of the precise nature and amounts of estrogens in blood

plasma concentration of free progestin fluctuated from a maximal level of 2 μg per milliliter to nondetectable levels (less than 0 2 μg per milliliter) progestin levels in relation to time following parturition up to 10 days showed no distinctive pattern or correlation with the presence or absence of lactation the results obtained on monkeys in this study were similar

Forbes (1955) compared progestin levels in blood drawn from the umbilical artery and vein just after delivery of the child but before the separation of the placenta The arterial level of free progestin was appreciably higher than the venous level in 16 of the 25 human pairs of samples thus obtained The free progestin values ranged from 0 3 to 7 5 μg per milliliter of plasma in the 25 arterial samples and from less than 0 2 to 5 0 μg per milliliter of plasma in the corresponding venous samples It would appear that more progesterone (progestin?) is moving from the fetus to the placenta than from the placenta to the fetus Forbes interpreted these data to indicate production of progesterone by the fetus and removal of the hormone by the placenta In support of this view he cited the evidence obtained by Hoffmann (1947) for the production of the hormone by the adrenal cortex of the human fetus in increasing amounts during the last months of pregnancy Ober (personal communication to Forbes 1955) and Zander and Solth (1953) have detected progestin in extracts of adrenal cortical tissue from human fetuses near term The possibility arises according to Forbes that not all the progesterone found in the placenta or released by it into the maternal circulation is actually synthesized by that organ

It might be pointed out that the above findings are at variance with those of Hoffman and Lam (1942 1948) (see p 419) who observed a pattern in the systemic level of progestin (McGinty test) in women also with those of Zander (1955) who found circulating progesterone (physicochemical estimates) levels to be higher in advanced human pregnancy than in early pregnancy

Rabbit Ewe Mouse The virtually cyclic fluctuations in circulating progestin described above with no really high levels at any time during pregnancy in human beings (or in the monkey) are in contrast with the following observations in animals

In the rabbit and ewe (Neher and Zarrow 1954 Zarrow and Neher 1955) systemic serum progestin levels follow a progressive course which is related to the stage of pregnancy and of the puerperium serial dilutions of the sera with physiological saline were employed

per milliliter of plasma In mice with intrasplenic implants progestin was present predominantly in bound form and it is noteworthy that the endometria of these mice showed no Hooker Forbes reaction indicating that the circulating bound progestin despite its high concentration was biologically inactive

A more extensive study on human pregnancy was subsequently made by Forbes (1951) The level of free progestin did not exceed 2 μg per milliliter of plasma in 2 women from the fifteenth week of pregnancy to normal term and there was at least one instance when no hormone (less than 0 2 μg) could be found in from one to seven successive weekly samples the plasma progestin levels were irregular and no pattern was discernible There is apparently no indication from these data of a progressive endogenous progesterone production with ad vancing pregnancy such as is reflected by the urinary excretion of pregnanediol (for summary data on the latter see Venning 1948) Similar results were obtained by Schultz (1953) in a study of systemic plasma progestin during the first trimester of human pregnancy Of the forty eight plasma samples which were obtained from 46 pregnant women (6 to 17 weeks after the last menstrual period) ten gave posi tive responses in the Hooker Forbes test Of these eight gave values below 2 0 μg one of 4 8 μg and one of 7 0 μg per milliliter no progestin (less than 0 2 μg per milliliter) was detected in the remaining samples There was apparently no correlation between the plasma progestin level and the stage of gestation Similar data were obtained by Forbes (1951) on plasma progestin levels in 3 pregnant rhesus monkeys

Klein and Ober (1952) found that the progestin concentration in peripheral venous blood of women in the third and fourth and also in the eighth and tenth months of pregnancy was significantly higher than that during the normal menstruating cycle These investigators also observed that the progestin concentration is higher at a given moment in uterine vein blood than in arm vein or uterine arterial blood of women in early or late pregnancy This observation lends support to the view that the placenta is an important source of pro gesterone in human pregnancy

Peripheral progestin levels during the puerperium were investigated by Forbes (1951) Plasma progestin in 1 woman following delivery was 1 5 μg per milliliter on the third day 1 7 μg per milliliter on the fifth and 0 2 μg per milliliter on the eighth A similar but more extensive study (Forbes 1953a) of 11 women in the puerperium followed The

too limited to support more than the statement that a fetal production of progesterone may occur in this species

Menstrual Cycle (Human Beings, Monkey)

Forbes (1950) determined plasma progestin levels during the course of the menstrual cycle in 4 women Free progestin rose to peaks of 1 7 to 5 2 μg per milliliter of plasma during the luteal phase of the cycle and then declined although in most cases progestin did not disappear until after the onset of the menses As in previous studies the level of bound progestin was very low (less than 0 5 μg) There was no apparent correlation between the onset of menstruation and the plasma progestin level It was clear that in all of the four donors studied there were instances when the menses began and continued for 24 hours or longer although appreciable quantities of free progestin were still circulating progestin was also detected prior to the time when ovulation was thought to have occurred Hoffmann and Lám (1948) similarly observed the serum progestin (McGinty test) to be maximal during the luteal phase of the cycle and it is also pertinent to recall that urinary pregnanediol excretion occurs at this stage of the cycle ceasing 1 to 3 days before menstruation (Venning and Browne 1937 Venning 1948) Buchholz *et al* (1954) undertook the arduous task of determining simultaneously the systemic progestin and the urinary pregnanediol levels and found that these follow approximately the same course during the menstrual cycle Hinsberg and Konrad (1954) independently arrived at similar conclusions maximal progestin values of 4 0 5 0 and 8 0 μg per milliliter of serum were observed and these declined just prior to menstruation serum progestin was minimal (less than 1 0 μg) during the menses Ober *et al* (1954) similarly observed progestin to be present in peripheral blood during the luteal phase of the cycle and that the progestin level declined before menstruation removal of the corpus luteum at the height of its functional activity resulted in a fall in the systemic progestin level to half its value within 24 hours

Hooker and Forbes (1949a) reported a progestin concentration of 6 0 μg per milliliter of plasma in the luteal phase of the menstrual cycle in a rhesus monkey In a subsequent study (Forbes *et al* 1950b) the level of free progestin was found to be low during and following menstruation high near the middle of the cycle low following this peak and high in the latter half of the cycle in one instance a progestin concentration as high as 7 7 to 8 9 μg per milliliter of plasma

for injection in the Hooker Forbes assay procedure In the rabbit (Zarrow and Neher 1955) the progestin level rose from a minimum of 0 3 to 1 0 μg per milliliter of serum at the time of mating to a maximum of 8 to 10 μg per milliliter at parturition Thereafter the first significant change a 10 to 50 per cent decrease in the progestin level occurred at 12 hours post partum By 24 hours after delivery the progestin level had returned to prepregnancy values of 0 3 to 1 0 μg and this level was maintained throughout the first 21 days of the lactation period Ovariectomy of rabbits at an advanced stage of gestation resulted in a marked decline in the progestin level to a value of 0 3 μg per milliliter of serum followed by abortion within 1 to 3 days In the ewe (Neher and Zarrow 1954) the progestin level rose progressively with advancing pregnancy to a maximum of 8 to 12 μg per milliliter of serum and declined rapidly following parturition Ovariectomy as early as the sixty sixth day of gestation did not interrupt the pregnancy and the progestin level remained high during the remaining half of the gestation period These studies suggest that the placenta is the major source of progestin during the last two trimesters of pregnancy in the ewe but that in the rabbit the ovary is the major source of the hormone Since a drop in progestin occurs in both species following parturition Zarrow and co workers do not regard as tenable the concept that delivery occurs as a result of a decreased level of progestin

Forbes and Hooker (1957) estimated plasma levels of free progestin during pregnancy in the mouse A biphasic curve was observed with maxima of about 8 μg per milliliter at about the eighth and fifteenth day of gestation and with minima during the first few days and at the twelfth day of gestation parturition occurred at the nineteenth day The progestin curve is thus different from that obtained in the pregnant rabbit (Zarrow and Neher 1955) or ewe (Neher and Zarrow 1954) From the foregoing studies on the progestin level in human beings and in animals at parturition Forbes and Hooker (1957) regard the relationship of progestin to the onset of labor to be obscure if indeed any relationship exists

Forbes (1955) compared progestin levels in umbilical arterial and venous blood in the goat The blood samples were obtained in advanced pregnancy prior to the separation of the placenta The free progestin levels ranged from less than 0 2 to 4 6 μg in the five arterial samples and from less than 0 2 to 1 0 μg per milliliter of plasma in the corresponding venous samples The author regarded the data as

Male Subjects The Role of the Adrenal Cortex and Testis as Sources of Progestin

Human Beings Lazo Wasem *et al* (1954) estimated the progestin level in men at 0 33 to 1 00 μg (average 0 77) per milliliter of serum The origin of the circulating progestin in men is probably the adrenal cortex

Bull Ram, Rabbit Hamster Squirrel Rat Circulating progestin levels (micrograms progesterone equivalents per milliliter of serum) were reported by Lazo Wasem *et al* (1954) as follows bull 0 33 to 1 00 (average 0 55) ram 0 33 to 1 00 (average 0 66) rabbit 0 00 to 1 00 (average 0 44) hamster 0 00 to 0 33 (average 0 20) squirrel 1 00 (average 1 00) rat 0 00 to 1 00 (average 0 33) In the 14 rats under study there was no change in the progestin concentration following castration thus it would appear that the testis of the rat is not a significant source of progestin In certain species the testis (and possibly the adrenal cortex) may be important in this respect since for example the bull is known to excrete large amounts of pregnanediol in the urine (Marker *et al* 1938) It has also been shown that progesterone serves as a metabolic precursor of androgens in surviving testicular tissue (Slaunwhite and Samuels 1956) but whether it is an obligatory intermediate accumulating in any appreciable amounts in the testis remains to be established

Adrenalectomy of castrated male rats is followed by the complete disappearance of progestin from the peripheral circulation (Lazo Wasem *et al* 1954) Additional evidence that the adrenal glands release progestin was obtained by Zarrow and Lazo Wasem (1955) as follows Castrated male and female rats were injected intramuscularly with 40 I U of corticotrophin the progestin values rose from a mean of 0 3 μg per milliliter of serum before treatment to 4 7 μg 2 hours after injection A maximum concentration of 8 0 μg was attained at 5 hours thereafter the progestin concentration declined reaching preinjection levels by 10 hours Similar results were obtained in experiments in castrated rabbits Epinephrine injected into castrated rats resulted in a rise of progestin to a maximum of 7 0 μg per milliliter of serum in 5 hours Exposure of castrated rats to cold produced a maximal progestin concentration of 2 7 μg per milliliter of serum at 12 hours Administration of chorionic gonadotrophin to castrated rats did not significantly affect progestin levels Incubation of bisected

was observed The levels of bound progesterone were low at all times An earlier study of a similar nature on the rhesus monkey was made by Allende (1940) who estimated that during the menstrual cycle of 1 of the 3 monkeys under study the amount of progestin in 1 ml of blood serum varied between a maximum of 0 25 to 2 5 μg and a minimum of 0 06 to 0 12 μg In 2 of the monkeys studied the progestin level was maximal on the tenth and eleventh day of the menstrual cycle and then gradually fell during the second half of the cycle The bioassay it will be noted was performed by intra uterine injection of alkali washed ether extracts of the serum into rabbits and the results were expressed on the McPhail scale

Estrous Cycle (Rabbit, Ewe)

Neher and Zarrow (1954) found that the circulating progestin level varied in a cyclic manner during the estrous cycle of the ewe a peak of 6 μg per milliliter of serum was reached during the luteal phase of the cycle and a minimum of 2 μg or less per milliliter of serum at the onset of the next heat period

Forbes (1953b) obtained evidence showing the secretion of progestin in the rabbit prior to ovulation Free progestin in the peripheral blood was observed to appear an average of 100 minutes after mating and of 64 minutes after intravenous administration of gonadotrophin whereas ovulation is known to occur 9½ to 10 hours after mating Maximal progestin levels in the blood samples drawn in the first 3 to 4 hours after mating or gonadotrophin injection ranged from 0 3 to 3 9 μg per milliliter of plasma The source of progestin in the absence of functional corpora lutea is uncertain In the pseudo pregnant rabbit progestin levels of 5 3 to 8 0 μg per milliliter of plasma were found (Hooker and Forbes 1949a)

Ovulatory Cycle (Fowl)

Fraps *et al* (1948a) found a progestin concentration of at least 0 33 μg per milliliter of plasma in the ovulating hen this finding is of interest as the authors point out because all previous attempts to identify luteal tissue or progesterone in the bird s ovary have led to either inconclusive or negative findings A progestin level as high as 1 0 to 3 0 μg per milliliter of plasma was observed in the non laying hen (Fraps *et al* 1948b)

but since the serum progestin level falls following castration in the pregnant rabbit (Neher and Zarrow 1953) it would appear that the adrenal gland is not an important source of progestin during gestation in this species

Turtle, Snake, Frog Fowl Lazo Wasem *et al* (1954) found progestin levels of 0 00 to 0 33 (average 0 16) 0 33 to 1 00 (average 0 66) and 0 00 to 0 33 (average 0 16) μg per milliliter of serum in the turtle snake and frog respectively Fraps *et al* (1948b) reported progestin concentrations as high as 1 0 to 3 0 μg in the cock but less than 0 33 μg per milliliter in the capon it appears that the testis is a source of progestin in fowl

PHYSICOCHEMICAL METHODS OF ESTIMATING PROGESTERONE IN BLOOD

The estimation of progesterone in blood and in other tissues requires (1) extraction of the tissue and fractionation of the extract and (2) physicochemical analysis of the purified fraction Such analysis is usually not specific for the progesterone molecule which need not be an obstacle provided that the fraction analyzed consists predominantly of progesterone In practice variable amounts of interfering impurities may be present these may be removed by further fractionation a gain which is offset by a corresponding loss of the hormone However one may calculate the precise extent of this loss in each instance by adding a known amount of radioactive progesterone in the initial extraction step and subsequently determining the radioactivity recovered in the final fraction the amount by weight of radioactive progesterone added must be negligible with respect to that of the radioinert endogenous progesterone present The addition of radioactive hormone may also serve as a guide in the form of an internal marker during the course of the fractionation

Extraction of Blood

At an early stage of extraction and fractionation a crude product free of protein and relatively free of neutral fat phospholipides and cholesterol may be obtained The procedures employed by Pearlman and Thomas (1953) follow A large volume of acetone is added to blood in order to precipitate proteins and phospholipides The filtrate is concentrated *in vacuo* to a small volume to remove most of the acetone and it is then extracted with ether The residue from the

adrenal glands of rats with corticotrophin gave a maximal concentration of 8 μg per milliliter of incubation fluid after 2 hours controls without corticotrophin gave 0 3 μg per milliliter These data clearly indicate a release of progestin from the adrenal glands both *in vivo* and *in vitro* by mechanisms which are also responsible for the release of adrenal cortical hormones There is no indication however of the precise chemical nature of adrenal progestin although it is well known that the adrenal cortex elaborates progesterone 17α-hydroxyprogesterone and other hydroxylated C_{21} steroids (reviewed by Lieberman and Teich 1953) Moreover the progesterone concentration (physico chemical estimation) in the adrenal venous blood of cattle sheep and pig is about ten to one hundred times greater than that in the arterial blood at a given moment (Balfour *et al* 1957) In the light of the recent report by Zarrow *et al* (1957) that 17α hydroxyprogesterone is sixty times as potent as progesterone in the Hooker Forbes test when Rockland Swiss mice are employed it is likely that the progestin released by the adrenal glands consists chiefly of 17α hydroxyprogesterone That the adrenal glands can produce physiologically significant amounts of progestin is also indicated by the observation by Lyons *et al* (1953) that deciduomata can be maintained with adrenocorticotrophin hormone (ACTH) in hypophysectomized-oophorectomized rats However one must be careful not to equate Hooker Forbes progestin with the progestin measured by the deciduomata test since the relative biological activities of the test compounds may differ considerably in the respective biological procedures However a product of the adrenal cortex namely desoxycorticosterone which is inactive in the McGinty or in the Hooker Forbes test does give rise to progestin (Hooker Forbes test) following its administration as the acetate to monkeys (*Macaca mulata*) according to Zarrow *et al* (1950) These investigators concluded that desoxycorticosterone is convertible to progesterone *in vivo* but in view of the uncertainty since their publication of the chemical nature of progestin (Hooker Forbes test) their conclusion is open to question Indeed in a similar study Lazo Wasem and Zarrow (1955) injected desoxycorticosterone acetate intramuscularly into rats and rabbits but referred to the progesterone like substance detected in increased amounts in peripheral blood as progestin (Hooker Forbes test) Klein and Ober (1952) extended this observation to human subjects

Zarrow and Lazo Wasem (1955) considered the adrenal gland as a possible source of the increased progestin observed during pregnancy

A simple countercurrent distribution of progesterone requiring only eight transfers in ordinary separatory funnels was described by Pearlman (1954a) The solvent system was petroleum ether 70 per cent methanol in which the partition coefficient (K) of progesterone is 0 33 A countercurrent distribution of progesterone in a Craig apparatus requiring twenty four transfers was described by Diczfalusy (1952) The solvent system was petroleum ether 34 5 per cent ethanol in which K (progesterone) is 3 55 This procedure was designed for the analysis of progesterone in placental tissue but it is undoubtedly applicable to blood extracts as well

Partition column chromatography of progesterone in blood extracts was described by Butt *et al* (1951) The supporting material was diatomaceous earth (Celite) the solvent system aqueous methanol n hexane A very high degree of resolution is attainable in partition column chromatography because such columns contain a relatively large number of theoretical plates which are analogous to the separatory funnels or tubes employed in countercurrent distribution The partition coefficients of progesterone and of certain other $\alpha\beta$ unsaturated ketonic steroids in various solvent systems are listed in Table I These constants (applicable in countercurrent distribution and in partition column chromatography) indicate that progesterone may be readily partitioned from many other $\alpha\beta$ unsaturated ketonic steroids

Although paper chromatography may or may not give the high resolution afforded in partition column chromatography it is a relatively simple procedure rapid and adequate for the purpose Its application to steroid analysis was introduced by Zaffaroni and Burton (1951) subsequently Edgar (1953a) Zander and Simmer (1954) and others devised chromatographic procedures especially suitable for progesterone estimation The Bush solvent system A (Bush 1952) consisting of petroleum ether (mobile phase) 80 per cent methanol (stationary phase) was found unsuitable by Zander and Simmer (1954) because the R_f value (0 85) of progesterone in this system indicated that the hormone was moving too close to the solvent front More appropriate R_f values of 0 73 0 68 0 51 and 0 38 were obtainable by using slightly modified solvent systems for example hexane 70 per cent methanol and by employing different grades of paper in ascending and descending chromatography Savard (1954) has reported on the mobility of a very large number of steroids including progesterone in a ligroin propylene glycol system in which the latter solvent is the stationary phase Although this paper chromatographic procedure has been widely employed in steroid analysis it appears not

ether extract is partitioned between petroleum ether and 70 per cent methanol cholesterol and neutral fat accumulate in the former phase progesterone in the latter The residue obtained from the aqueous methanol phase is treated with Girard s reagent T to obtain the ketonic fraction but this step is not essential particularly if counter *current distribution or similar partition procedures are to be subse* quently employed

The extraction procedures employed by Zander and Simmer (1954) in their extensive studies on blood progesterone are a modification of those devised by Butt *et al* (1951) The plasma or blood is added to a large volume of an ethanol ether (3 1) mixture The supernate obtained on centrifugation is concentrated *in vacuo* diluted with water and extracted with ethyl acetate The residue obtained from the ethyl acetate extract is dissolved in warm 70 per cent methanol chilled to −15°C for 18 hours and centrifuged at high speed in the cold The supernate after dilution with water is extracted with petroleum ether the residue from the latter extract is subsequently subjected to partition chromatography

An alternative procedure (Zander and Simmer 1954) was designed to obviate the use of a high speed cold centrifuge The ethanol ether supernate in the foregoing procedure is extracted after concentration with benzene instead of with ethyl acetate The benzene extract is filtered and the filtrate evaporated to dryness the residue is extracted with 70 per cent methanol the latter diluted with 2 volumes of water and extracted with petroleum ether The residue obtained from the latter extract is further purified as above

A procedure suitable for rapid extraction of large volumes of plasma (for example 500 ml) was described by Short (1958a) Sufficient sodium hydroxide is added to plasma to give a final concentration of 0 5 gm of sodium hydroxide per hundred milliliters of plasma This permits extraction with ether without the formation of troublesome emul sions The ether extract after washing with water is evaporated the residue is partitioned between petroleum ether and 70 per cent meth anol the residue from the latter phase is purified by paper chroma tography

Fractionation of Blood Extracts Countercurrent Distribution Partition Column Chromatography and Paper Chromatography

The partitioning procedures commonly employed in purifying the hormone include countercurrent distribution partition column chromatography and paper chromatography

Ultraviolet Spectroscopy Reynolds and Ginsburg (1942) described a procedure for the quantitative estimation of progesterone in serum by ultraviolet analysis the extraction procedure was essentially that employed by W M Allen for the extraction of the hormone from sow corpora lutea It will be noted that the intense absorption exhibited by progesterone at 240 millimicrons is not specific for the hormone molecule *per se* but is due to the presence of an $\alpha\beta$ unsaturated ketonic group thus a solution of 19 1 μg of progesterone per milliliter of ethanol has an optical density of 1 00 at 240 millimicrons in a light path of 1 cm According to Loofbourow (1943) photoelectric measurements of the optical density (d) are most precise when d = 0 43 *the error being of the order of* $\pm$ *1 per cent* The limits to the preci e estimation of progesterone are thus self-evident With the aid of an electron photomultiplier attached to a Beckman Model DU spectrophotometer one may expect a considerable gain in sensitivity permitting the analysis of a much smaller volume for example 0 4 ml *of progesterone solution (light path 1 cm) in a microcuvette* As little as 0 1 μg of progesterone in all may thus suffice for analysis but even this is a rather high requirement because only about 0 1 μg of the hormone is present per milliliter of peripheral blood in human pregnancy However smaller amounts of the hormone may be detected *by preparing an appropriate ketonic derivative such as the* bisthiosemicarbazone (Bush 1953 Pearlman and Cerceo 1953 Talbot *et al* 1955) which exhibits an absorption maximum at 301 millimicrons molecular extinction coefficient 40 250 the isonicotinic acid hydrazone of progesterone (Sommerville 1957) has also been employed

Infrared Spectroscopy Infrared analysis of the $\alpha\beta$ unsaturated ketone detected by ultraviolet spectroscopy in extracts of human pregnancy blood established its identity as progesterone (Zander 1955) The application of infrared analysis as a quantitative tool is however very limited because of the small amount of hormone present in blood

Polarography Polarographic estimation of progesterone in blood extracts as the water soluble ketonic derivative obtained with Girard s reagent T was described by Butt *et al* (1951) this method is not specific although about equally sensitive as ultraviolet analysis Purification of the extracts was by *partition column chromatography* It is noteworthy that impurities often present in progesterone fractions which exhibit nonspecific absorption in the ultraviolet and thus interfere with ultraviolet analysis may be expected not to interfere in polarographic analysis

TABLE I Partition of Progesterone and Certain Other α,β Unsaturated Ketonic Steroids

Compound	P (partition coefficient)*			R (paper chromatography)†		
	1	2	3	4	5	6
Progesterone	0 33	3 55	0 51	0 73	2 3	1 ?8
Δ4 Cholesten 3-one	3 1	—	—	—	9 5	—
7 keto cholesterol	1 8	—	—	—	—	—
Δ4 Androstene 3,17 dione	0 07	—	0 17	0 39	1 7	1 18
Testosterone	0 05	0 28	—	0 15	0 33	0 64
Desoxycorticosterone	0 03	0 21	0 05	0 20	0 45	1 00
Cortisol	—	<0 02	—	0 0	—	—
Δ4 Pregnen-20α-ol 3 one	—	—	—	0 38	0 24	0 88
Δ4 Pregnen 20β-ol 3 one	—	—	—	0 38	—	0 99
17α Hydroxyprogesterone	—	—	—	—	0 28	0 73

*Column 1 system petroleum ether 70% methanol from the data of Pearlman (1954a)
Column 2 system petroleum ether 34 5% methanol from the data of Diczfalusy (195?)
Column 3 system n hexane 70% methanol calculated from the da a of Butt et al (1951)
†Column 4 R_f values system n hexane 70% methanol (stationary phase) descending from the data of Zander and Simmer (1954)
Column 5 system ligroin propylene glycol (stationary phase) from the data of Savard (1954) The constants are R_T values related to a reference standard
Column 6 system toluene propylene glycol (stationary phase) from the d ta of Zander et al (1958) The constants are related to desoxycorticosterone (R 1 00)

to have been applied to the estimation of blood progesterone However a toluene propylene glycol system has recently been employed by Zander *et al* (1958) in paper chromatography to resolve mixtures of Δ4 pregnen 20α-ol 3-one and Δ4 pregnen 20β ol 3 one which accompany progesterone in certain organ extracts The mobility on paper of progesterone and several other α β unsaturated ketonic steroids in various solvent systems is indicated in Table I

Estimation of Progesterone

The progesterone content of the purified fractions obtained in the various procedures outlined above may be determined by the following physicochemical methods

hormone was encountered in the procedure Final identification of the hormone was made by infrared analysis of the combined eluates of the progesterone spots on the paper chromatograms following re chromatography Circulating progesterone levels were appreciably lower during the first half of pregnancy quantitative estimation was not possible although as much as 50 ml of individual specimens of peripheral blood were available The elevated levels of circulating progesterone in advanced pregnancy thus reflect an increased endoge nous production of the hormone

The progesterone concentration in uterine vein blood was found by Zander and Munstermann (1954) to average 0 26 μg of progesterone per milliliter of blood during the tenth month of pregnancy 0 14 μg during the fifth month and 0 0 μg during the third month From the difference in progesterone levels in uterine vein blood and in peripheral blood and from the rate of flow of uterine blood it was estimated that the daily placental production of progesterone is at least 190 mg in late pregnancy (Zander and Munstermann 1956) In a study by Pearlman (1957b) who administered known amounts of tritium labeled progesterone to 3 women in advanced pregnancy and subsequently determined the specific activity of the urinary pregnane 3α 20α diol it was calculated that about 250 mg of endogenous proges terone is produced daily by the body From the above data and from other indications (see discussion by Pearlman and Cerceo 1952) it appears that the placenta is a major source of progesterone in advanced pregnancy in women

Progesterone levels in umbilical cord blood were determined by Butt *et al* (1951) five individual specimens were analyzed and found to contain 1 3 0 3 0 0 0 0 and 0 0 μg of progesterone per milliliter of blood Zander and Munstermann (1954) found an average of 0 34 μg of the hormone per milliliter of blood (4 cases) and an average of 0 53 μg per milliliter of plasma (3 cases) in umbilical vein blood ob tained immediately after birth Short (1958a) found an average of 0 86 μg of progesterone per milliliter of plasma in umbilical cord blood (4 cases) The progesterone concentration in pooled placental blood was found by Pearlman and Thomas (1953) to be 0 4 μg of hormone per milliliter of blood One may infer from these data that an appreciable portion of placental progesterone enters the fetal cir culation and that the circulating progesterone level in the fetus is perhaps higher than that in the mother Further study is required to elucidate these relationships

Fluorometry Bush (1952) observed that certain $\alpha\beta$ unsaturated ketonic steroids including progesterone fluoresce in ultraviolet light following treatment of the hormone on the paper chromatogram with aqueous sodium hydroxide and heat A method for the quantitative estimation of progesterone (and other Δ^4 3 ketosteroids) based on the same principle but employing the reagent potassium t butoxide in t butyl alcohol in a test tube reaction was recently developed by Abelson and Bondy (1955) as little as 0 01 μg of the steroid can be detected However its application to the estimation of progesterone in blood appears not to have been reported

Qualitative Tests for Progesterone Various nonspecific color reactions which may be useful in detecting minute amounts of progesterone have been reviewed by Zander and Simmer (1954) A solution of progesterone in sulfuric acid exhibits maximum absorption at 295 millimicrons (Axelrod 1953) This absorption spectrum is not unique for progesterone but it may serve to distinguish the hormone from certain other related Δ^4 3 ketosteroids at least 10 μg of the hormone is required Kalant (1958) has recently reported on the chromogenic and fluorogenic reactions of various steroid hormones including progesterone in concentrated sulfuric acid and in 100 per cent phosphoric acid Ultraviolet reflex photography is very useful in locating progesterone and other $\alpha\beta$ unsaturated ketonic steroids on paper chromatograms (for example see Bush 1952a)

PROGESTERONE BLOOD LEVELS (PHYSICOCHEMICAL ESTIMATIONS)

Pregnancy

Human Beings Haskins (1950) found by ultraviolet analysis less than 0 1 μg of progesterone per milliliter of pregnancy blood Butt *et al* (1951) confirmed this observation employing polarography Low peripheral blood progesterone levels were also reported by Edgar (1953b) and Zander (1955) both investigators employed paper chromatography and ultraviolet analysis Zander (1955) thus analyzed nine pooled samples of peripheral venous blood varying from 195 to 426 ml each which were obtained from women in the latter half of pregnancy an average of 0 142 μg (range 0 039 to 0 268) of progesterone per milliliter of plasma was found corresponding to 0 078 μg of progesterone per milliliter of blood since there is little or no progesterone in red blood cells a loss of about 20 per cent of the

was observed on the day (about 280 days) before calving there were *0 001 to 0 004 μg of progesterone per milliliter of plasma* It was not possible to identify progesterone by infrared analysis because not enough hormone was available It is rather remarkable that the circu lating progesterone level during pregnancy in the cow should be so very much lower than that in man The daily amount of endogenous progesterone elaborated in the pregnant cow is not known nor has the source of the hormone been clearly established in this species Pro gesterone is not detectable in cow placental extracts (Pearlman 1954b) however the placenta of the cow may be contributing some proges terone to the circulating pool of the hormone since removal of the corpus luteum after about 200 days of pregnancy does not necessarily induce an abortion (*cited by Short* 1958b)

Edgar (1953b) found 0 5 to 2 0 μg of progesterone per milliliter of *ovarian vein blood in the pregnant ewe In a more extensive study* Edgar and Ronaldson (1958) found that the progesterone concentration in ovarian vein blood rose from nondetectable levels (less than 0 15 μg) during the first few days of pregnancy attained a maximum level of about 2 μg per milliliter during the fifth week and then declined to nondetectable levels at lambing (twentieth week) Progesterone was not detectable in peripheral vein blood during pregnancy in this species this is also true in the cow and mare (Edgar 1953b) The ovary thus appears to be an important source of progesterone during gestation In blood from the vein draining the pregnant horn of the uterus progesterone was detected in low concentrations between the ninth and eighteenth weeks of pregnancy in only 6 of 143 cases This suggests that the uterine contents are not an important source of progesterone The authors point out however that since the blood flow through the uterus greatly increases during pregnancy a low concentration might represent a relatively large secretion of proges terone It is interesting to compare these findings with those of Neher and Zarrow (1954) *who reported maximum levels of 8 to 12 μg of* progestin (Hooker Forbes test) in the peripheral circulation of preg *nant ewes* (see p 425) *It is apparent that only a very small fraction* 2 per cent or less of the circulating progestin can be accounted for as *progesterone It is also interesting that the* peripheral progestin level remained high following ovariectomy at the sixty sixth day of gestation *despite the importance of the ovary* as noted above as a source of progesterone

The highly active gestagens Δ^4 pregnen 20β ol 3 one and Δ^4 pregnen 20α ol 3 one (see p 421) could not be detected by Zander *et al* (1958) in peripheral pregnancy blood even when employing as much as 50 ml of plasma yet a mixture of the 20 hydroxy steroids is present in human placental extracts (Zander *et al* 1958) in a ratio of 1 0 part to 5 8 parts of progesterone in which the 20α-hydroxy epimer is predominant (Zander *et al* 1957) (In an independent study Salhanick *et al* 1956 had identified Δ^4 pregnen 20α ol 3 one in human placental extracts) A mixture of the 20α and 20β hydroxy steroids (of undetermined ratio) was also detectable in extracts of human placental blood of corpora lutea of pregnancy and of menstruation and of ripe Graafian follicles (Zander *et al* 1958 Zander 1958) these appear on the paper chromatograms as substances more polar than progesterone with sim ilar absorption at 240 millimicrons (see Table I) Gorski *et al* (1958) found 0 79 μg of Δ^4 pregnen 20β ol 3 one and 4 33 μg of progesterone per gram of bovine ovary (nonpregnant cows or heifers) In view of these findings the above 20α hydroxy and 20β hydroxy steroids may be regarded together with progesterone as gestagens of natural occur rence

The high progestin level in peripheral pregnancy blood reported by Hooker and Forbes (1949a) thus cannot be accounted for as proges terone — the progesterone equivalent concentration (Hooker Forbes bioassay) is about fiftyfold that of progesterone (physicochemical esti mation) — nor can it be accounted for as the newly discovered gesta gens Δ^4 pregnen 20β ol 3 one and Δ^4 pregnen 20α-ol 3 one which as mentioned are not detectable in peripheral blood Zander (to be pub lished) has suggested (1) that other compounds with significant progestin activity may be present in pregnancy blood but are as yet unrecognized and (2) that a synergism between known compounds may exist which enhances the biological activity of blood extracts Forbes has emphasized in this connection that the Hooker Forbes test is probably best regarded as a method of detecting certain com pounds and not as a test for progestational activity

Cow, Ewe Mare Progesterone levels in the peripheral venous blood of cows during the course of gestation were determined by Short (1958b) Large volumes of individual specimens of blood for example 1 liter were available for analysis paper chromatography and ultra violet spectroscopy were employed The hormone concentration ranged between 0 0074 and 0 0098 μg of progesterone per milliliter of plasma from days 32 to 256 of gestation but thereafter a marked decrease

Klopper and Michie 1956) From the rate of urinary excretion of pregnane 3α 20α diol in the preinjection control period one can *calculate the average rate of endogenous progesterone production* This estimate is subject to certain reservations since it is assumed that the rate of hormone metabolism is uniform throughout the post injection collection period and that the rate is identical with that in the pre injection control period It should also be noted that *the dose of hormone administered usually exceeds the physiological level*

(2) A more reliable and accurate estimate of the average rate of hormone production may be obtained by a procedure similar to that above but employing radioactive progesterone One need determine only the specific activity of the urinary pregnane 3α 20α-diol since the amount and specific activity of the hormone injected are known Most of the assumptions made in applying method (1) apply here as well although a tracer dose of the hormone may be administered however the analytical errors are considerably reduced It is assumed that the urinary metabolite is derived entirely from progesterone which may not necessarily be the case

Thus for example progesterone 16 H^3 was administered to pregnant women (Pearlman 1957b) and the endogenous production thereby calculated

(3) The instantaneous rate of progesterone secretion may be calculated from the arteriovenous differences in the hormone concentrations of the endocrine organ in question and the rate of flow of blood through that organ Experimentally this procedure is difficult to perform and is of limited application It may also be subject to serious technical inaccuracies Thus for example the rate of placental production of progesterone in women was determined in essentially this manner by Zander and Munstermann 1956 (see p 437)

(4) The instantaneous progesterone production rate may also be determined by injecting a trace amount of radioactive hormone and *observing the rate of decline of the specific activity of the hormone* in serial blood samples obtained at stated intervals It must be emphasized that it does not suffice for this purpose to determine simply the radioactivity in the blood as this may be only partly due to the hormone but that it is necessary to determine also the concentration *of radio inert endogenous hormone (See the next section* for an exposition of general principles of tracer methodology see Siri 1949)

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Rate of Disappearance of Progesterone from the Blood Stream

Rate of Turnover The determination of the turnover time of endogenous progesterone in circulating blood is based on the following theoretical premises The amount by weight of radioactive hormone injected intravenously must be very small in relation to the total amount of endogenous circulating hormone if the system is not to be disturbed a steady state must be maintained in which the hormone blood level and the rate of endogenous hormone production remain constant A first order reaction may be expected wherein the rate of declining concentration of radioactive hormone is directly proportional to its concentration (C) at any given instant (t)

$$\frac{dC}{dt} = -kC$$

The integrated equation is

$$C = C_0e^{-kt}$$

or

$$2\ 3 \log \frac{C_0}{C} = kt$$

where C_0 is the initial concentration of radioactive hormone and C the concentration after t minutes and k is a constant called the turnover rate The value of k is obtained most readily when $C = 0\ 5C_0$ for then the last equation above becomes

$$k = \frac{0\ 693}{t_{\frac{1}{2}}}$$

in which $t_{\frac{1}{2}}$ is designated as the half life time The turnover rate k is the fraction r/M of the total amount of hormone replaced per unit time M is the total amount of hormone (labeled and unlabeled) in the vascular compartment r is the actual rate at which the hormone leaves the vascular compartment and hence at the steady state is the rate of production of endogenous hormone The reciprocal of the turnover rate (k) is called the turnover time (T) and is equal to the fraction M/r

The rate of disappearance of trace amounts of progesterone 4 C^{14} from peripheral blood following intravenous injection of the hormone into men and women was recently determined by Sandberg and Slaunwhite (1958) The half life time ($t_{\frac{1}{2}}$) of the radioactivity in the ether or chloroform extracts of plasma was 20 minutes during the initial equilibration period and subsequently 90 minutes These constants may or may not

be identical with the half life time of progesterone because the radio activity due to the hormone in such extracts was not determined presumably unconjugated progesterone metabolites were also present in this fraction Conjugated progesterone metabolites detected in the plasma were predominantly glucuronidates and to a minor extent sulphates The radioactivity in the conjugated fraction of plasma was considerably higher than that in the unconjugated one also the rate of disappearance of radioactivity in the conjugated fraction was considerably lower

The rate of disappearance of radioinert exogenous progesterone from the peripheral circulation has also been determined However the half life time of exogenous hormone cannot be applied to calculate the turnover time of the endogenous hormone It will also be noted that in such experiments the initial blood level of the administered hormone far exceeds the physiological concentration In the pioneer studies of Haskins (1950) about 10 mg of progesterone was injected intravenously into each rabbit and the hormone concentration estimated by ultraviolet analysis of the plasma extracts Later Zarrow *et al* (1954) carried out similar experiments except that progesterone was estimated by the Hooker Forbes test In both studies the hormone concentration was plotted against time if one recasts the data as a semilogarithmic plot a linear relationship is observed Only one major component of the curve is apparent and its slope is constant as physiological hormone levels are approached However the slope is ill defined in the initial equilibration period which is very short The data furnished by the two groups of investigators were thus found to be in good agreement The half life time was 4 9 and 11 5 minutes in intact and hepatectomized rabbits respectively (Haskins 1950) and 3 3 and 9 1 minutes respectively (Zarrow *et al* 1954) The liver thus appears to be an important factor influencing the rate of disappearance of progesterone (see p 445) In similar experimental studies on man the amount of radioinert progesterone administered was likewise far above the physiological level the sampling of blood was too infrequent to permit a precise determination of the rate of disappearance of the hormone Thus Haskins (1950) injected 100 mg of progesterone intravenously into a pregnant woman with ante partum eclampsia and found a progesterone concentration of 3 8 and 1 6 μg per milliliter of plasma after 1 and 8 minutes respectively Zander (1954) injected 200 mg of progesterone intravenously into menopausal or ovariectomized women the hormone concentrations were 1 44 0 116 and less than 0 1 μg per milliliter of blood after 3 to 5 minutes 2 hours

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jections of progesterone-4 C^{14} according to a recent study by Plotz and Davis (1957) (2) The amount of progesterone in pregnancy urine is a very small fraction of the total amount produced in the body in marked contrast with the prodigious quantities of the urinary metabolites

That the liver is an important site of progesterone metabolism has been well established (see review by Pearlman 1948) Thus for example progesterone is biologically inactive when implanted into the spleen (Forbes and Hooker 1949) the hormone under these circumstances must pass through the liver before entering the systemic circulation It has also been shown that hepatectomy markedly delays the disappearance of exogenous progesterone from the peripheral circulation in rabbits (Haskins 1950 Zarrow *et al* 1954) In a case of infectious hepatitis in a woman in the sixteenth week of pregnancy the progesterone concentration was very high about 0 4 μg per milliliter of blood (Butt *et al* 1951) Since endogenous hormone production in early pregnancy is normally low the turnover time of blood progesterone may have been very much prolonged in this case

The kidney may also be important in progesterone inactivation Forbes *et al* (1950a) obtained evidence of renal inactivation of circulating progestin (Hooker Forbes test) in the rhesus monkey by determining progestin levels in renal arterial and venous blood during the latter half of the menstrual cycle The free progestin concentration was 0 9 to 8 3 (average 4 0) μg per milliliter of arterial plasma and 0 2 to 2 6 (average 1 6) μg per milliliter of renal plasma the arterial levels were higher than the venous in each instance (3 pairs) From the arteriovenous difference in progestin concentration (average 2 4 μg per milliliter) and assuming a kidney passage of 150 milliliters of plasma per minute one arrives at a loss of about 360 μg of progestin per minute or 500 mg daily This seems rather high in view of an estimated daily production of 250 mg of progesterone in advanced human pregnancy (Pearlman 1957b) However as discussed above the chemical identity of blood progestin is not known at present

THE TRANSPORT OF PROGESTERONE IN BLOOD

The interaction of progesterone and plasma proteins is discussed at length by Antoniades and Slaunwhite (Chapter XIV) It suffices to mention here that there is some evidence for such interaction but that only part of the progesterone present in the plasma participates

and 24 hours respectively These results clearly indicate that exogenous progesterone rapidly disappears from the blood stream in man

The turnover time (T) may also be viewed as a constant relating the peripheral blood concentration (c) to the rate (r) of endogenous progesterone production at the steady state Since the total amount of hormone (M) in blood is a product of its concentration (c) and the total blood volume (V) $T = cV/r$ To calculate turnover time by the latter method the concentration (c) of radioinert endogenous progesterone in peripheral blood is required There is not available at present a physicochemical method which is sufficiently sensitive or specific for the quantitative determination of the low progesterone blood levels which obtain during the menstrual cycle or during early pregnancy However quantitative estimates of peripheral progesterone levels in advanced human pregnancy have recently been made notably by Zander (1955) From these data and those on the rate of endogenous progesterone production in advanced pregnancy (Pearlman 1957b) the turnover time of progesterone in the blood was calculated to be about 3 3 minutes (Pearlman 1957a) This is a crude approximation but one which is none the less instructive For example one would expect the peripheral hormone level to fall precipitously in the event of a marked decline in hormone secretion as for example following parturition It has not been established whether the turnover time varies with the endocrine status of the individual In this connection it is pertinent to note that the intermediary metabolism of progesterone 16 H^3 in human pregnancy was found not to differ markedly from that in nonpregnancy in the absence of luteal function (Pearlman 1957b)

Metabolism and the Rate of Turnover The very short turnover time of progesterone in the blood stream in human pregnancy is probably due largely to the rapid rate at which the hormone is metabolized This may be inferred from these observations (1) Very little of the hormone as such accumulates in the body The progesterone in body fat detected by Kaufmann and Zander (1956) although appreciable in amount appears to be but a small fraction of the daily endogenous production of the hormone In animal experiments in which trace amounts of progesterone 21 C^{14} were administered the hormone did not accumulate to any significant extent in any of the organs or tissues examined (Barry *et al* 1952) However large amounts of radioactivity — 18 20 and 34 per cent of the injected dose — accumulated in the maternal fat of pregnant women who had received intramuscular in

jections of progesterone-4 C^{14} according to a recent study by Plotz and Davis (1957) (2) The amount of progesterone in pregnancy urine is a very small fraction of the total amount produced in the body in marked contrast with the prodigious quantities of the urinary metabolites

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The protein binding which is of a weak and reversible nature is probably an important factor in the transport of the hormone in the body A further study of these factors may also contribute to an under standing of the mechanism of hormone action

The extent of binding of endogenous progesterone and its metabolites to plasma proteins is not known at present (for studies on bound progestin see p 423) However some data having an impor tant bearing on this problem have been obtained in metabolism exper iments in which progesterone-4 C^{14} was injected intravenously into human subjects (Sandberg and Slaunwhite 1958) A portion of the radioactive steroid like material both free and conjugated in the plasma was associated with certain protein fractions obtained in the Cohn fractionation procedures Fractions IV 1 (γ globulin and cho lesterol) IV-4 (α and β globulins and metal combining globulin) and especially V (bulk of albumin) were responsible for the transport of the unconjugated material (Sandberg *et al* 1957)

PROGESTERONE METABOLITES IN PERIPHERAL BLOOD

Apart from progesterone a study of its metabolites in circulating blood is not without interest for it may shed further light on the manner in which progesterone undergoes biological inactivation Also it is conceivable that progesterone is converted into other biologically active compounds hitherto unrecognized which may make their ap pearance in the blood stream in small amounts Certain progesterone metabolites which are apparently totally lacking in biological activity may nevertheless be of physiological importance in view of the report by Velardo (1957) that pregnanediol stereoisomers antagonize the action of progesterone in decidual development a finding consistent with the concept of Hisaw *et al* (1954) that the metabolic alterations of the reproductive tract are the sum total expression of all the hormones and/or their metabolites in a physiological system at a specific time

Certain progesterone metabolites which have been isolated from organ extracts or from urine are probably also present in blood but in amounts too small to be measured by conventional techniques Information as to the nature of such metabolites in blood may perhaps be more readily obtained in metabolism experiments entailing the intravenous administration of trace amounts of radioactive proges terone followed by isotopic dilution analysis of the plasma radio

activity In a study of this type by Sandberg and Slaunwhite (1958) on the metabolic fate of [4 C^{14}] progesterone in human subjects the plasma radioactivity was found chiefly in the conjugated fraction (mostly glucuronidates) the identity of the individual metabolites was not reported Davis *et al* (1956) injected intramuscularly [4 C^{14}] progesterone into pregnant women radioactivity appeared in the plasma *but the nature of the radioactive material was not ascertained*

PROGESTERONE METABOLISM SOME MAJOR ASPECTS

For purposes of orientation particularly in discussing progesterone metabolites in the peripheral circulation a brief review of major aspects of progesterone metabolism may be helpful (for an exposition of the subject see reviews by Pearlman 1948 1952 Venning 1952 Lieberman and Teich 1953 Zander 1957b)

Organs which are major contributors to the pool of circulating hormone in man are the ovary and the placenta although progesterone is formed in the adrenal cortex and serves as a metabolic precursor of the adrenal cortical hormones the amount of progesterone released by this organ is normally very small

The major end product of progesterone metabolism in man is pregnane 3α 20α diol It appears in large quantities in human pregnancy urine as the glucuronidate accompanied by smaller amounts of pregnan 3α-ol-one 20 glucuronidate and pregnane 3 20 dione also present but in minor proportions are other related C_{21} steroids for example allopregnan 3β ol one 20 and allopregnane 3α 20α-diol

The major pathways of progesterone metabolism are shown in Figure 2 Hydrogenation of the double bond between carbon 4 and carbon 5 results in complete loss of biological activity of the molecule

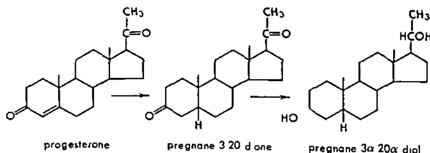


FIGURE 2 Progesterone metabolism a major route

this metabolic transformation is probably chiefly responsible for the rapid inactivation of the hormone which occurs mainly in the liver Hydrogenation of the carbonyl group at carbon 20 yields the potent naturally occurring gestagens Δ^4 pregnen 20α ol 3 one and Δ^4 pregnen 20β ol 3-one Hayano *et al* (1954) observed that progesterone on incubation with corpora lutea of cattle is converted to Δ^4 pregnen 20β ol 3 one Berliner and Wiest (1956) and Wiest (1956) demonstrated an extrahepatic metabolism of progesterone C^{14} in rats to Δ^4 pregnen 20α-ol 3 one

The importance of 17α hydroxyprogesterone as a naturally occurring gestagen in man may be negligible despite its potency in this connec tion (Salhanick *et al* 1957) as the 17α acyl derivative This compound whose presence in extracts of the adrenal cortex was detected very early (see review by Reichstein and Shoppee 1943) arises from progesterone in this organ 17α Hydroxyprogesterone has recently also been identified in extracts of human follicles and corpora lutea its con centration per gram of tissue was estimated to be 1 7 μg also present were progesterone (15 5 μg) a mixture of Δ^4 pregnen 20α-ol 3-one and Δ^4 pregnen 20β ol 3 one (2 4 μg) and Δ^4 androstene 3 17 dione (0 6 μg) (Zander 1957a 1958) It may be of pertinent interest to mention that in the recently demonstrated conversion *in vitro* of progesterone to androgens in the ovary (Solomon *et al* 1956) and in the testis (Wotiz *et al* 1955) 17α hydroxyprogesterone is formed presumably as an intermediate

Acknowledgment

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CHAPTER XIV

The Binding of Steroid Hormones and Their Metabolites by Plasma Proteins

Harry N Antoniades, William H Daughaday, *and* W Roy Slaunwhite, Jr

SECTION 1 THE BINDING OF STEROID HORMONES AND THEIR METABOLITES BY PLASMA PROTEINS *IN VIVO*

Harry N Antoniades

Binding of steroid hormones and their metabolites by blood proteins

Endogenous steroid hormones

Intravenously injected steroid hormones

Binding of steroid hormones and their metabolites by plasma protein fractions

Endogenous steroid hormones

Intravenously injected steroid hormones

Possible nature of protein steroid interactions

Role of the liver in protein steroid binding

Biological significance of protein steroid binding

SECTION 2 THE BINDING OF ESTROGENS ANDROGENS AND PROGESTERONE BY PLASMA PROTEINS *IN VITRO*

W Roy Slaunwhite Jr

Introduction

Interaction of proteins with small molecules

Reversible binding

Equilibrium dialysis

Estrogens

Testosterone

Progesterone

Comparative binding of steroids

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William H Daughaday

The binding of corticosteroids by albumin

Corticosteroid binding globulin

Corticosteroid binding by plasma protein fractions

Identification of corticosteroid binding globulin by electrophoresis

Properties of corticosteroid binding globulin

Alterations of activity of corticosteroid binding globulin

Conclusions

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BINDING OF STEROID HORMONES AND THEIR METABOLITES BY BLOOD PROTEINS

The first attempt to study and demonstrate the binding of steroid hormones to serum proteins and serum protein fractions was carried out *in vitro* by Brunelli (1935) He demonstrated that estrogen added to serum did not dialyze through a collodion membrane He assumed that this was due to binding of the estrogen to serum proteins In an attempt to find which serum protein fraction was responsible for the binding of estrogen the serum proteins were separated in two fractions (1) the globulin fraction precipitated by diluting the serum with distilled water at acid pH and (2) the supernatant fraction characterized as the albumin fraction The estrogenic activity was present in what Brunelli characterized as the globulin fraction This fractionation of the serum proteins was a crude one However the work on the binding of steroid hormones to plasma and plasma proteins which followed Brunelli s experiment under *in vivo* and *in vitro* conditions basically resembles this first demonstration More advanced methods are in use at present with better controlled conditions

These methods can be grouped in the following categories (1) precipitation of blood or plasma and serum proteins with organic solvents followed by determination of the steroid hormone in the protein free supernatant fluid and protein precipitate (2) protein precipitation *through salting out techniques or with organic protein precipitants* with subsequent examination of the precipitate and protein free supernatant fluid for the presence of the steroid hormone (3) dialysis of whole blood plasma or serum the dialyzates and the protein fractions inside *the membrane being analyzed for the hormone and (4)* ultrafiltration of blood serum the protein free filtrates and the protein fractions being analyzed for the presence of the steroid hormone

Endogenous Steroid Hormones

Investigations on the binding of steroid hormones by blood plasma or serum proteins are inconsistent in their findings Early studies by Haussler (1936) suggesting that part of the endogenous estrogen in the blood stream of pregnant and nonpregnant mares was associated with the protein fraction of serum were not confirmed by Muhlbock (1937) when he repeated these studies under similar experimental conditions More recent attempts to demonstrate protein-steroid bind

Section 1

The Binding of Steroid Hormones and Their Metabolites by Plasma Proteins *in Vivo**

Harry N Antoniades

There is a growing interest for a better understanding of the binding of steroid hormones by plasma proteins and of the biological significance which may exist through such a protein steroid interaction and association Experimental data suggest that steroid hormones in blood are associated with plasma proteins Interpretation of these data however depends on the experimental conditions employed to demonstrate and study the protein steroid complexes In *in vitro* experiments primarily between an unconjugated steroid and a protein presumably of high purity the system may be under control and the conditions in general are favorable for quantitative studies of the protein steroid interaction (see Slaunwhite Section 2 Daughaday Section 3) *In vitro* experiments however may not represent the physiological state At present the nature of steroid hormones in the blood stream is unknown as well as the nature of the proteins which transport the individual steroid hormone What holds true for *in vitro* studies may not be true for *in vivo* experimental conditions where the metabolites of the steroid hormone the complex protein system or even possible participation of enzymes may effect the protein steroid hormone binding However *in vitro* experiments are undoubtedly helpful at present in our efforts to understand some of the problems involved in the study of the steroid transport mechanism in blood

On the other hand *in vivo* studies devoted to isolation and characterization of the protein steroid complexes although closer to the physiological state may be misleading if the fractionation methods employed cause denaturation of the proteins and disruption of the protein steroid complexes Binding studies following intravenous administration of steroid hormones to human beings are affected by a variety of factors and it is not clear to date whether these studies represent the physiological state It is important therefore to take all these factors into consideration and interpret the experimental data under the special conditions employed for each study thus avoiding generalization of the results

* Part of this work was supported by grants from the National Institutes of Health Public Health Service (RG–5525) and from the National Foundation

hormones and their metabolites in plasma following intravenous ad ministration of steroid hormones with radioactive label to human beings dissociate from the protein upon addition of acetone to plasma even at low temperatures (Sandberg *et al* 1957) However ultra filtration studies and dialysis experiments indicated that practically all the steroid was bound to plasma proteins

Studies on the binding of endogenous estrogen to blood proteins which employed various methods without the use of organic solvents have been reported by Rakoff *et al* (1943) and by Szego and Roberts (1946 1947) The binding of estrogen by serum proteins was demon strated by Rakoff *et al* (1943) by several methods (1) salting out with sodium sulfate (2) precipitation with sulfosalicylic acid and (3) ultrafiltration The protein free supernatant fluid following precipi tation of the plasma proteins with sodium sulfate or sulfosalicylic acid was found inactive Hydrolysis of the supernatant fluid did not change the results The protein precipitate contained practically all the activity accounted for in the whole serum These findings were interpreted as suggesting that estrogen in human pregnancy serum is intimately bound to serum proteins Further evidence concerning the binding of estrogen from pregnancy serum was obtained from ultrafiltration studies The filtrate was free of estrogenic activity indicating association with the serum proteins

Ammonium sulfate precipitation of serum proteins followed by prolonged hot alcohol-ether Soxhlet extraction of the protein pre cipitate showed that approximately two-thirds of the total estrogen *in the blood* was bound to proteins (Szego and Roberts *1946 1947*) One third of the estrogen was recovered in the protein free supernatant fluid The latter amount represented the unbound portion of the steroid The authors suggested that this portion is in the conjugated form since it was extractable with ether only after acid hydrolysis The binding of estrogen by plasma proteins was also demonstrated by Szego and Roberts with ultrafiltration studies and with the use of acetone and alcohol-ether precipitation of the blood proteins of normal and pregnant cows normal and gonadotrophin injected rab bits and pregnant women The estrogen was extracted from the proteins by weak hydrolysis with 0 1 normal sodium hydroxide fol lowed by extraction with ether The latter observation however which indicates a strong binding of the endogenous estrogen to plasma proteins is in contradiction with the finding that blood estrogen dialyzes quantitatively through sausage casing (visking 25/32 inch

ing in blood also vary Rakoff *et al* (1943) and Szego and Roberts (1946 1947) reported that estrogen is bound to plasma proteins However Bischoff *et al* (1954) did not reconfirm Szego and Roberts results and Hooker and Forbes (1949) concluded that only a small portion of progesterone in blood is bound to plasma proteins The reason for these discrepancies may lie to some extent in the inadequacy of the techniques which were employed for the demonstration of the protein steroid binding (see p 463) The more we learn about proteins and protein complexes and their preservation (see Chapter 2) the greater is the concern for a more critical review of the conditions employed in the studies of the protein steroid binding

Precipitation of blood or serum proteins with the use of large volumes of organic solvent was the usual procedure in the majority of these studies Haussler for example precipitated the serum proteins with large volumes of acetone (1 part serum 5 5 parts acetone) The protein precipitate was extracted with acetone and ether and tested for estrogenic activity Part of the estrogenic activity of the serum was present in the protein precipitate despite the extraction with acetone and ether This was interpreted as an indication that this portion of the hormone was either in a form insoluble in acetone and ether and not necessarily bound by proteins or that it was closely associated with the serum proteins Haussler accepted the latter interpretation primarily on the basis of reported observations at that time that the portion of cholesterol in plasma not extractable with ether was associated with the proteins and was extractable only by boiling with alcohol The fact that Mulhbock (1937) was unable to show the presence of estrogen in the protein fraction in studies similar to those of Haussler may be due to the fact that he used even larger volumes of acetone for the precipitation of the blood proteins (1 part serum 10 parts acetone) These studies in general have little value in providing information on the state of estrogen in blood Under the experimental conditions employed disruption of the protein steroid binding is expected as well as extended denaturation of the serum proteins

The presence of estrogen (Bischoff *et al* 1954) progesterone (Hooker and Forbes 1949) and androgens (Hilmer and Hess 1950) in the protein free supernatant fluid following precipitation of serum proteins with large volumes of organic solvent is not surprising and does not necessarily indicate that these steroids are unbound in the blood stream As will be discussed (see p 465) exogenous steroid

binding of steroids by blood proteins derived from ultrafiltration and dialysis may better represent the natural state of the protein steroid complexes than data obtained from experiments which employ organic solvents especially in large volumes as blood protein precipitants

Intravenously Injected Steroid Hormones

Sandberg *et al* (1957) reported that no or very little radioactivity was present in the acetone or ethanol and ethanol chloroform precipitate of human plasma collected 15 to 240 minutes after the intravenous administration of estrone 16 C^{14} cortisol-4 C^{14} and testosterone 4 C^{14} However dialysis and ultrafiltration studies indicated that the administered hormones were practically all bound to plasma proteins These data support the suggestion that large volumes of organic solvents probably dissociate the protein steroid complexes The precipitate in the above experiments was free of radioactivity whereas ultrafiltration and dialysis experiments demonstrated that almost 90 per cent of the radioactivity was bound to plasma proteins following administration of estradiol 16 C^{14} testosterone-4 C^{14} progesterone 4-C^{14} cortisol 4 C^{14} corticosterone-4 C^{14} and 4 C^{14} Δ^4 androsten 11β ol 3 17-dione

Dialysis equilibrium experiments were carried out by Daughaday (1956a) on human plasma obtained two hours after the ingestion of oral cortisone acetate (75 mg) The results indicated that 94 per cent of the 17 hydroxycorticosteroids and 65 per cent of the 17 hydroxy corticosteroid glucuronides were loosely bound to plasma proteins

Data on the amount of unconjugated and conjugated steroid hormone bound to plasma protein have been reported by Sandberg *et al* (1957) and Antoniades *et al* (1957a) Following intravenous administration of C^{14} steroid hormones the plasma was fractionated by Cohn s cold ethanol method 6 (Cohn *et al* 1946) The radioactivity present in the plasma fractions was regarded as the portion bound to plasma proteins The radioactivity present in the remaining supernatant fluid (supernatant fluid V) was regarded as representing the unbound steroid The unconjugated steroids were extracted from the fraction three times with 30 ml of freshly distilled chloroform The conjugated steroids were extracted twice with large volumes of ethanol (5 to 10 volumes) The residue dissolved in water was hydrolyzed and extracted consecutively by three procedures (1) hydrolysis with β glucuronidase and extraction three times with ether (2) continuous extraction with ether at pH 1 0 and (3) refluxing with 15 volumes

diameter) when blood or plasma is dialyzed at 2°C for 36 to 72 hours against 5 volumes of distilled water

The experimental data on the binding of steroid hormones by whole blood or serum proteins are but few and even fewer provide valuable information on the binding of estrogen in blood The experimental conditions employed for the demonstration of the protein steroid complexes must meet at least two requirements (1) preservation of the protein steroid complexes and (2) preservation of the blood and serum constituents (Details on these requirements are presented in Chapter II by Oncley) Various factors may affect the studies on the binding of steroids to plasma proteins such as pH temperature ionic strength the presence of other ions and molecules which may compete for binding sites of the protein The presence of organic solvents also affects the protein steroid binding The systems described in these studies employed mainly large volumes of organic solvents as protein precipitants Early studies (Haussler 1936 Muhlbock 1937) probably were performed at room temperature whereas in later studies the addition of the organic solvent was carried out at 0° to 3°C However the statement that the proteins in the precipitate were undenatured in the presence of an excess of organic solvent is doubtful On the contrary in the presence of an excess of organic solvent it is expected that a significant part of the proteins will probably be denatured The highest ethanol concentration used in the fractionation procedures developed by Cohn and his associates (see Oncley Chapter II) was 40 per cent at −5 C Even under mild conditions (18 per cent ethanol pH 5 2) sensitive proteins such as the α_1 lipoproteins are readily denatured

Axelrod and Zaffaroni (1954) observed that the rate of dialysis of blood corticosteroids was highly increased when they added an organic solvent phase to the dialysis equilibrium system Sandberg *et al* (1957) have shown that the presence of 40 per cent ethanol at −5°C dissociates appreciable amounts of estrone from the albumin-estrone complex *in vitro* It is therefore possible that the use of organic solvents as protein precipitants in steroid binding studies is misleading

The effect of pH on the binding of cortisol by plasma proteins was reported by Daughaday (see Section 3) He noticed that at pH 5 2 in acetate buffer the cortisol binding globulin undergoes *irreversible* changes Details on the effect of pH and ionic strength on the protein steroid binding are discussed elsewhere (Slaunwhite Section 2 Daughaday Section 3) At present it seems probable that data on the

binding of steroids by blood proteins derived from ultrafiltration and dialysis may better represent the natural state of the protein steroid complexes than data obtained from experiments which employ organic solvents especially in large volumes as blood protein precipitants

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per cent hydrochloric acid and extraction three times with ether (Sandberg and Slaunwhite 1956) Table I shows that the radioactivity present in the supernatant fluid V varies from 24 to 34 per cent for estrone estradiol testosterone and progesterone and from 60 to 75

TABLE I Distribution of Bound and Unbound Radioactivity in Human Plasma Following Injection of C^{14} Labeled Steroids

St roid	Bo d (F acti ns I to V)			Unbound (Sup rnatant Fluid V)		
	Total	Co jug ted	U co jug ted	T tal	C jug ted	U jug ted
E tro e[1] c/mi /100 ml † pe cent	2155 66	1440 67‡	715 33‡	1110 34	500 45‡	610 55‡
E tradiol[2] c/min./100 ml per ce t	3335 66	2130 64	1250 36	1720 34	340 20	1380 80
Testo terone[3] c/mi /100 ml p ce t	10320 76	9625 94	695 6	3200 24	26 0 83	530 17
Proge terone[4] c/min./100 ml per c t	3523 70	3300 94	2 3 6	15 0 30	1130 75	390 25
Co ticoste ne[5] c/mi /100 ml per e t	4827 27	3310 69	1517 31	12480 73	6840 55	5640 45
Co ti ol[6] c/m /100 ml pe e t	3714 40	2223 60	1491 40	5720 60	2420 42	3300 58
Δ[4] Andro t [7] c/m n./100 ml per c t	2963 29	2554 86	409 14	7250 71	5700 79	1550 21

From Anto iade t l J. B. l Ch m 9 1071 1957
†Coun p mi te p 100 ml f plasma.

1 Blood llect d 45 min fte I.V i j ti n f estro 16 C^{14} (3,000 0 0 /m)
2 Blood llect d 20 mi ft I.V i j ti f t adiol 17 16-C^{14} (1 100 000 /m)
3 Bl d ll ted 0 ml aft I.V j ti n f t to t -4 C^{14} (800 000 /m)
4 Blood llect d 15 m ft I.V jecti f p g t ro 4 C^{14} (1 680 000 /min)
5 Blood ll ted 20 m ft I.V inj ti n f tico t ro 4 C^{14} (1 530 000 /min.)
6 Bl d ll ted 120 min. afte I.V i ject f i I 4 C^{14} (" 970 000 /m)
7 Blood ll ted 10 min. ft I.V injecti n f 4 C^{14} Δ^4 andr t 11β -ol 3 17 di (3 040 000 /m n.)
‡P tages ar al l ted ising th l ln th T tal col m as 100 p ent

per cent for corticosterone cortisol and Δ^4 androsten 11β-ol 3 17 dione and their metabolites

The values for the unbound steroid are probably higher in the fractionated plasma than in the unfractionated plasma due to partial dissociation of the steroid protein complexes under the conditions of the fractionation procedure *especially for Fractions IV 4 (40 per cent* ethanol pH 5 8) and V (40 per cent ethanol pH 4 8) Values for the bound steroid calculated in these experiments are much lower than values reported from ultrafiltration and dialysis techniques (Daughaday 1956b Sandberg *et al* 1957) Another factor which must be

taken into consideration in this study is that the supernatant fluid V from Cohn s fractionation contains approximately 1 per cent of the total amount of the plasma proteins and is composed primarily of albumin α globulins and β globulins and glucoproteins A portion of the steroids and their metabolites in the supernatant fluid may also be bound to these proteins

BINDING OF STEROID HORMONES AND THEIR METABOLITES BY PLASMA PROTEIN FRACTIONS

Endogenous Steroid Hormones

Since Brunelli s (1935) attempt to locate the plasma fraction responsible for the binding of estrogen plasma protein fractionation techniques and knowledge of the nature of plasma proteins have been greatly improved Szego and Roberts (1946) examined various fresh undialyzed Cohn fractions for estrogenic activity The activity present in the plasma was recovered nearly quantitatively in Fraction III 0 which contains the bulk of the β lipoproteins Traces of activity were present in Fractions IV 1 (α globulins and cholesterol) and V (albumin) The conclusion that estrogen is bound to β lipoproteins is not necessarily correct since Fraction III 0 also contains appreciable amounts of a mixture of proteins such as albumin and α globulins and β globulins (see Chapter II) The authors suggested that on the basis of differential solubility the estrogen in Fraction III 0 is estriol and can be removed from the protein only by hydrolysis However contrary to this suggestion dialysis for 72 hours does remove the bulk of the estrogen from the protein

Bischoff *et al* (1954) fractionated pregnancy plasma by Cohn s method 6 The II + III precipitate which contains Fraction III 0 was found inactive The activity was present in the II + III supernatant fluid which contains Fractions IV 1 IV 4 V and VI No estrogenic activity was found in dried Fractions III 0 IV 1 and IV-4 even up to doses equivalent to 2000 mg By dialyzing between human serum and human serum albumin Bischoff and Stauffer (1957) have recently shown that estriol and estradiol added to normal serum and the endogenous estrogen of last trimester serum migrate from serum to albumin at a rate and amount comparable to the migration from albumin to albumin Other serum proteins showed no indication of competition with the albumin These findings were interpreted by Bischoff and Stauffer as indicating the importance of albumin in the

transport of estrogen in serum Studies following intravenous administration of steroid hormones (Antoniades *et al*, 1957a b Sandberg *et al* 1957) also indicated the importance of albumin in the steroid transport mechanism in blood (see pp 465–470) In the latter studies the participation of the α globulins has also been suggested in the transport of steroid hormones and their metabolites in the blood stream

Gardner (1954) examined various Cohn fractions for 17 ketosteroids by the Zimmermann reaction following chromatography on Florisil The activity was present primarily in Fraction IV 4 although Fractions IV 1 and V were also active Fractions I II and III were found practically inactive The supernatant fluid of Fraction V was not tested The amount of 17 ketosteroids recovered in the fractions was much lower than the activity accounted for in the whole plasma The loss of activity was possibly due to the age of the samples which were dried and stored for years before examination for 17 ketosteroids

Examination of Cohn s fractions by Sandberg *et al* (1957) for 17 ketosteroids showed a considerable variation in the concentrations of the 17 ketosteroids among the various fractions and within any single fraction prepared from different plasma pools The differences were probably due to diversity of sources of the human blood and the age of the samples at the time of the examination Fresh fractions yielded higher concentrations of 17 ketosteroids The results reported by Sandberg *et al* (1957) for the distribution of endogenous estrogen activity in various Cohn fractions tested for estrogenic activity in castrated female rats are not conclusive A larger number of animal tests are necessary in order to obtain statistically significant results

During the course of their studies on the distribution of endogenous estrogen in plasma protein fractions McArthur *et al* (1957) observed that the results from the biological assay depended greatly on the condition of the fractions Estrogenic activity in these studies was determined by the Astwood (1938) technique The plasma fractions were prepared from fresh normal pooled plasma and from plasma donated by pregnant women in the last trimester of pregnancy using cold ethanol methods 6 and 9 (Cohn *et al* 1946 Oncley *et al* 1949) Fractions administered without dialysis produced toxic effects a large number of animals died shortly after administration especially of Fraction V and supernatant fluid V Limited dialysis (3 to 4 hours) which removed the excess salt and ethanol greatly reduced the toxic effects to the animals During these studies about 35 fractions were

tested in 350 animals The bioassay was affected by the salt and ethanol concentration of the fractions and probably by the protein concentration and the protein composition *In view of these observations* a re-examination of the conclusions which are based on the bioassays of endogenous estrogen in concentrated plasma protein fractions may be necessary

Intravenously Injected Steroid Hormones

Two hours after the intravenous injection of 100 mg of crystalline estrone or estrone sulfate into male subjects (Antoniades *et al* 1957a) 500 ml of blood was collected through cationic exchange resin (see Pennell Chapter I) and the separated plasma was fractionated by cold-ethanol methods 6 and 9 (see Oncley Chapter II) The bioactivity was consistently present in Fraction V (albumin) and supernatant fluid V which probably represents the unbound portion of the estrogen Part of the activity was also present in Fraction IV 1 which contains α-globulins and cholesterol and also albumin (Fig 1) The albumin present in Fraction IV 1 was precipitated with anti human albumin horse serum in order to determine whether or not the activity present in this fraction was due to the contaminating albumin In three out of five experiments the immunoprecipitate was found active At the same time however activity was present in the immunosupernate thus indicating that both albumin and α-globulins may participate in the binding of the injected hormone

Binding studies following intravenous injection into human beings of steroid hormones were extended to a large number of steroids with the use of C^{14} labeled steroid hormones (Sandberg *et al* 1957 Antoniades *et al* 1957) Following intravenous administration of radioactive steroid hormones to human subjects plasma Fractions IV 1 and V appear to be the fractions most important in the transport of steroids and their metabolites Figures 2 and 3 show that Fraction V is the predominant carrier for six of the seven conjugated steroids and for three of the seven unconjugated steroids Fraction IV 1 is the predominant carrier for two unconjugated steroids and one conjugated steroid while unconjugated cortisol is found equally in the two fractions

The distribution of steroids and their metabolites per gram of protein (Figs 4 and 5) again indicates Fractions V and IV 1 to be the predominant carriers of steroids Of the two Fraction IV 1 is the more active component in this respect being the most active carrier

PLASMA

I
F b i g

II III

IV 1
α Gl bulins
h l t rol
alb mi
t gen

IV 4
α Gl bul
β m tal mbini g
gl bulin

V
Alb mi
t g

Supernatant Fl id V
estrog

II IIIw
γ Gl buli
tc

III O
β Lipop t ins
alb mi

Anti hum lb min
h rum

Precip tate
Album
est g

S pernata t Fl id
Gl b li
h l t rol
tr g

Dext an II t

Anti human alb m
h rum

P ecip tat
β Lipop t in

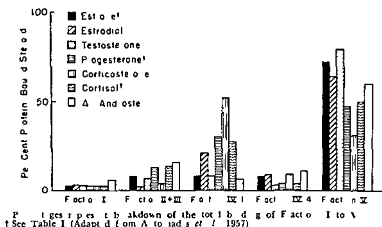
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β Gl b l ns
lbum

P ecipit te
Alb mi

S p natant Fluid
β Lipop t in
β gl b lin

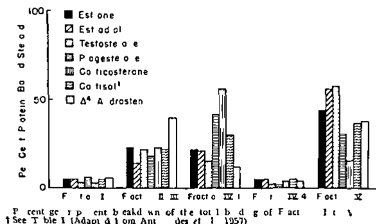
FIGURE 1 Distribution of infused estrone in human plasma (From Antoniades *et al* 1957)

for six of the seven unconjugated steroids and for four of the seven conjugated Unconjugated Δ^4 androsten 11β 3 17 dione is unique in that it associates most actively with Fraction II + III



P t ges r p es t b akdown of the tot l b d g of F act o I to V
† See Table I (Adapt d f om A to iad s *et l* 1957)

FIGURE 2 Per cent of binding of C14 steroid hormones (conjugated) to human plasma fractions



P cent ge r p ent b eakd wn of the tot l b d g of F act I t V
† See T ble I (Adapt d f om Ant des *et l* 1957)

FIGURE 3 Per cent of binding of C14 steroid hormones (unconjugated) to human plasma fractions

Purdy *et al* (1959) also reported that two hours after the intravenous injection of estradiol 16 C14 the largest amount of radioactivity was found in the supernatant fluid of Cohn Fraction IV I This fraction contains Fractions IV 1 V and the supernatant fluid V The estrogen

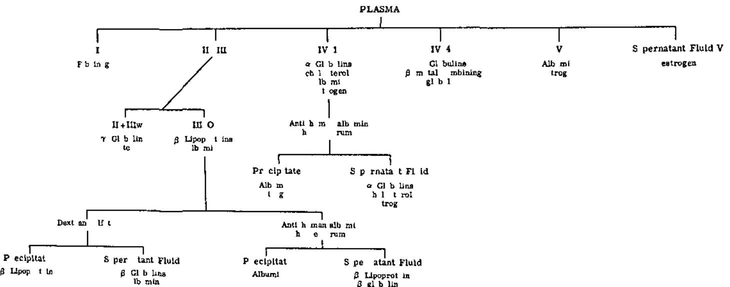


FIGURE 1 Distribution of infused estrone in human plasma (From Antoniades *et al* 1957)

method of fractionation The plasma fraction responsible for the binding of cortisol was the fraction containing the albumin and α- and β globulins (SPPS) The plasma globulin precipitate (PGP) fraction which contains primarily fibrinogen β lipoproteins and γ globulins was practically free of radioactivity However it is not certain whether or not zinc fractionation techniques are superior to those of the cold ethanol method since zinc ions compete with certain steroids such as testosterone and cortisol for binding sites of human plasma albumin *in vitro* (Slaunwhite *et al*)

The extent to which studies following intravenous administration of steroid hormones represent the physiological state is not clear as yet Both the concentration of the administered steroid hormone and the interval between the injection of the steroid and blood collection may be important for the interpretation of the results After administration of 100 mg of estrone and immediate collection of the blood all plasma fractions were contaminated with estrogen

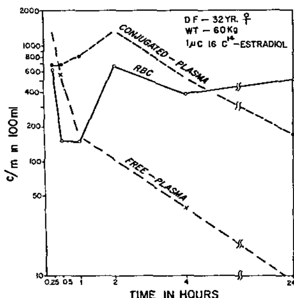
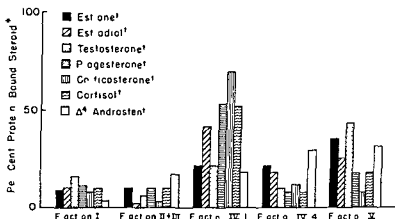


FIGURE 6 Levels of radioactivity of free and conjugated steroids in plasma and total radioactivity in erythrocytes following the injection of estradiol 16 C^{14} intravenously Free steroids refer to chloroform extractable compounds the conjugated steroids to chloroform or ether extractable steroids following β glucuronidase hydrolysis and extraction at pH 1 for 48 hours *The radioactivity in the erythrocytes (RBC) was that extracted with ethanol without subsequent hydrolysis* Note the high levels of radioactivity in the erythrocytes as late as 24 hours after the injection (From Sandberg *et al* 1957)

in the supernatant fluid IV 1 was found to be primarily estrone sulfate Purdy *et al* (1959) suggested that estrone sulfate is the major human circulating estrogen Estrone glucosiduronate in these studies was present in much smaller amount



* Percentages represent breakdown of the total binding of Fractions I to V
† See Table I (Adapted from Antoniades *et al* 1957)

FIGURE 4 Per cent of binding of C^{14} steroid hormones (conjugated) per gram of protein in plasma fractions



* Percentages represent breakdown of the total binding of Fractions I to V
† See Table I (Adapted from Antoniades *et al* 1957)

FIGURE 5 Per cent of binding of C^{14} steroid hormones (unconjugated) per gram of protein in plasma fractions

Fractionation of plasma proteins by equilibrating plasma with zinc (Surgenor *et al* 1959) after intravenous administration of cortisol 4 C^{14} showed results similar to those obtained with the cold ethanol

though to keep in mind that the problems involved in the study of the nature of the binding of steroids with proteins are numerous and that the interpretation of the results probably involves more than one structural element of the proteins and steroids which participate in these interactions

In the estimation of Szego and Roberts (1946) estrogen is bound to human plasma β lipoproteins in a hydrophilic esterified form The nature of the linkage to protein is such that hydrolysis with 0 1 normal sodium hydroxide was necessary to remove the bulk of the estrogen from the protein The value of this observation is confused however by the report that the estrogen was removed quantitatively from the complex by dialysis From solubility studies of steroid hormones in protein solutions Bischoff and Pilhorn (1948) suggested that the steroid is probably oriented in the solvent by secondary valence bonds of the albumin and other serum constituents However Bischoff *et al* (1954) following the observation that after acetone precipitation of human pregnancy serum the activity was present in the acetone protein free supernatant fluid and not in the protein precipitate suggested that the dispersion of estrogen in protein solution follows the classic laws of distribution based on solubility However the use of large volumes of organic solvent in order to demonstrate protein steroid binding may be misleading

Reversible complexes of steroids with human and bovine serum albumin have been studied extensively (see Slaunwhite Section 2 Daughaday Section 3) Quantitative studies of free energies of binding *in vitro* between unconjugated C^{14} labeled estrone estradiol testosterone progesterone corticosterone and cortisone and human serum albumin indicated a rather loose connection between the steroids and the albumin Eik Nes *et al* (1954) presented data on the binding of a large variety of steroids by bovine serum albumin which suggest polar and van der Waals interactions between the steroids and this protein Participation of the protein charged groups in the binding of unconjugated testosterone by bovine albumin has been indicated from the observation that methyl orange thiocyanate and cations compete with testosterone for the binding sites of albumin (Schellman *et al* 1954) Competition of anions with uncharged organic molecules for binding sites of albumin suggested (Klotz and Ayers 1952) that cationic side chains of the albumin molecule may be involved in the interaction of anions and possibly of neutral organic molecules with the albumin Levedhal and Bernstein (1954) demonstrated that the blocking of the

(Antoniades *et al* 1957a) When the blood was collected 2 hours after the administration of the crystalline estrone or estrone sulfate the activity was located primarily in Fractions IV 1 and V

Figure 6 demonstrates the effect of time on the concentration of unconjugated and conjugated steroid in human plasma following intravenous administration of estradiol 16 C^{14} The amount of un conjugated estradiol rapidly decreases soon after the injection of the hormone The amount of conjugated steroid however increases 1 to 4 hours after the injection The distribution of the radioactivity in fractions prepared from plasma collected at various intervals may differ since the conjugated steroid may bind to different proteins than those to which the unconjugated steroids bind Another con sideration in these studies is the system of separating the plasma proteins from one another One can assume that at least two and probably three of the variables employed in the separation of plasma proteins by the cold ethanol method of fractionation i e pH dilu tion and ethanol concentration could affect dissociation of loosely bound steroid from plasma proteins (Antoniades *et al* 1957b)

POSSIBLE NATURE OF PROTEIN-STEROID INTERACTIONS

The nature of the forces responsible for binding steroid hormones and their metabolites to plasma proteins is unknown Suggestions have been made that the binding of steroids by proteins may involve van der Waals forces hydrogen bonding electrostatic forces or other forms of binding The nature of binding depends on the stereochemical configuration of the steroid and the specific steric structure of the in dividual protein so that the reactive groups of the protein are avail able to those of the steroid Experiments thus far have been carried out primarily *in vitro* where usually unconjugated steroids and puri fied proteins are employed It is desirable at this point to state again that what holds true for *in vitro* studies may not be true for the binding of the endogenous steroids since their nature and the nature of the proteins which bind the individual steroid are as yet unknown

Turner (1954) in his excellent review on the physical and chemical properties of the steroids related to protein binding summarizes some of the steroid properties which may be associated in some way with their binding by proteins Interaction of steroids with substances simpler than proteins may be suggestive of the possible nature of their binding with the more complicated protein molecules It is necessary

tionship was also observed by Sandberg *et al* (1957) who indicated that the less polar steroids and the phenolic steroids are bound in greater quantities than are the corticosteroids Westphal and Ashley (1958) made a similar observation for the interaction of Δ^4 3 ketosteroids with human serum albumin bovine albumin β lactoglobulin human globin and casein However this relationship does not hold true for *other proteins such as lysozyme chymotrypsin chymotrypsinogen and* trypsin In these proteins the strength of interaction with progesterone cortexone and cortisol was found in direct proportion to the number of polar groups present in the steroid These proteins contain a larger amount of aliphatic hydroxy groups than the former proteins Westphal and Ashley (1958) suggested that the formation of hydrogen bonds between the hydroxy groups of the steroid and the aliphatic hydroxy side chains of the protein in these cases can be considered an effective counterbalance to the interaction of water molecules with the hydroxy groups of the steroids In the competition with the water molecules the proteins with more aliphatic hydroxy groups are better able to form complexes with the more polar steroids Daughaday (1958b) observed that nonradioactive corticosteroids may compete with cortisol 4 C^{14} and corticosterone 4 C^{14} for binding sites of human plasma However competition experiments between corticosterone and estrone progesterone and testosterone progesterone and estrone and testosterone and corticosterone indicated that there is no competition among these steroids for binding human serum albumin (Sandberg *et al* 1957) Solubility studies were performed in order to determine the possible range of steroid concentrations that could be used in these studies This observation may suggest that the nature of interaction for individual molecules both anions and neutral organic molecules may differ from one to another due to the heterogeneity of the binding sites of the protein (Karush 1950)

Interpretation of experimental data on the nature of the binding of steroids to proteins must take into consideration the structural elements for both the steroid and the protein which effect the binding The affinity of proteins for steroids may be affected in general from differences of positional isomers and stereochemical isomers and from the influence of certain functional groups and structural changes of the steroid molecules (Slaunwhite Section 2 Daughaday Section 3) Conjugated steroids for example which are highly polar molecules and at certain pH s are ionic may bind to different proteins from that of the unconjugated steroids (see Table I Figs 2–5)

ε amino acids of bovine serum albumin by selective acetylation (44 per cent and 88 to 92 per cent acetylation) does not influence the binding of testosterone to bovine serum albumin This indicated that at least in the binding of the uncharged testosterone the cationic side chains of the protein do not participate in the binding

Schellman *et al* (1954) on the basis of competition studies between testosterone and thiocyanate methyl orange and cations through dialysis equilibrium techniques and partition analysis suggested that the binding of testosterone to bovine serum albumin is a polar interaction The presence of the equally effective polar carbonyl and hydroxyl groups of the steroid being separated by about 20 angstroms may suggest an ion dipole interaction

The binding of anions and neutral organic molecules increases substantially as the pH in the system increases (Klotz and Ayers 1952) This holds true for the binding of testosterone to bovine serum albumin (Schellman *et al* 1954) and of estrone and progesterone to human albumin (Sandberg *et al* 1957) This may suggest that certain groups of albumin such as tyrosine may be involved in the binding the small molecule acting presumably as the acceptor of a hydrogen bond The suggestion that tyrosine groups may be present at the protein sites becoming accessible as the pH increases is supported by the observation that iodinated albumin in which the diiodotyrosine phenolic group becomes ionized at pH 9 thus losing its hydrogen donor character shows a great loss of affinity for organic molecules (Klotz and Ayers 1952) Levedahl and Perlmutter (1956) presented evidence that one molecule of testosterone is released when the phenolic groups of two tyrosine residues of bovine albumin are ionized However Oyakawa and Levedahl (1958) reported that iodination of the tyrosine groups of both human and bovine serum albumin in the 2 and 6 positions did not alter the binding characteristics of the albumin Evidence for the participation of the tyrosine groups in the binding of testosterone was obtained by ketenization of the phenolic hydroxyl groups of tyrosine Ketenization reduced the binding of testosterone by the albumins studied It was suggested (Oyakawa and Levedahl 1958) that testosterone binds in a configuration continuous and parallel to the ring structure of the tyrosine side chains coupled through the hydroxyl configuration of the residue

Eik Nes *et al* (1954) reported that the strength of binding of steroids by bovine serum albumin bears an inverse relationship to the number of polar groups of the steroid and its water solubility A similar rela

complexes with steroid hormones by the addition of various steroid hormones to human plasma *in vitro* and in the absence of liver has been reported by various investigators (Sandberg *et al* 1957 Migeon *et al* 1959 see also Sections 2 and 3 of this chapter) Studies with the use of plasma fractionation techniques (Sandberg *et al* 1957) and paper electrophoresis (Daughaday 1956c Slaunwhite and Sandberg 1959) also indicated that α-globulins and albumin were primarily responsible for this *in vitro* binding of steroid by plasma proteins without the participation of liver

Sandberg *et al* (1957) on the basis of their data indicated that the liver does not seem to play an important role in the evolution of a blood protein steroid complex although the liver proteins may bind strongly the radioactive estrone present in the system They suggested that a significant part of the estroprotein may not represent binding of estrone 16 C^{14} by the serum proteins but may merely be the in filtration of the serum by proteins from the liver with great affinity for the binding of estrogen They substantiated their suggestion with the following observations

(1) Liver tissue slices were incubated with estrone 16 C^{14} in a phos phate buffer without serum A considerable number of counts were associated with the proteins in the buffer these proteins having in filtrated the phosphate buffer medium from the liver (2) A large amount of radioactivity was present in the liver three times as much radioactivity when the liver was incubated with buffer than with serum This difference is probably due to the fact that when the liver is incubated with serum a considerable amount of estrone 16 C^{14} is bound by serum proteins thus making the steroid unavailable for liver proteins (3) After the injection of estrone 16 C^{14} in a rat the liver was removed and slices were incubated with Krebs Ringer phosphate buffer for 2 hours Twenty per cent of the total radioactivity in the liver was present in the supernatant buffer The radioactivity was due to the infiltration of liver proteins which bound metabolites of the radioactive estrone

Steroid hormones other than estrogen were bound by liver proteins Corticosterone 4 C^{14} testosterone 4 C^{14} and cortisol-4 C^{14} following incubation with rat liver tissue slices in Krebs Ringer phosphate buffer showed that a protein steroid complex was formed with these steroids Cortisol-4 C^{14} was the least avidly bound by the liver proteins in the supernatant fluid (Sandberg *et al* 1957)

Riegel and Mueller (1954) in their studies of the binding of radio

ROLE OF THE LIVER IN PROTEIN-STEROID BINDING

Participation of the liver in the binding of steroid hormones by blood proteins was suggested by Szego (1953) Incubation of estrone 16 C^{14} or estradiol 16 C^{14} with rat liver tissue slices *in vitro* in a homologous serum medium showed the presence of radioactivity in the serum and liver proteins Paper electrophoresis and cold ethanol fractionation of rat serum or serum albumin following incubation with rat liver tissue slices in the presence of estrone 16 C^{14} or estradiol 16 C^{14} indicated that the radioactivity was associated with the albumin fraction In the absence of liver tissue slices the radioactivity on paper electrophoresis remained close to the origin presumably unbound The latter experiment however is not conclusive Westphal *et al* (1956) and Daughaday (1956c) demonstrated that special conditions are necessary in order to produce migration of the radioactive unconjugated steroid added to serum on paper electrophoresis Without these conditions (equilibrium dialysis of the serum steroid complex against the electrophoresis buffer) the unconjugated steroid is adsorbed on the paper and remains close to the origin

The data presented above were interpreted as suggesting that the liver tissue catalyzes the binding of estrone 16 C^{14} and estradiol 16 C^{14} specifically to the albumin of a homologous serum medium (Szego and Roberts 1956) The estroprotein formed *in vitro* in the presence of liver tissue slices appeared as a glucuronoside complex associated with a protein carrier It was suggested that the liver is probably the site of formation of endogenous estroprotein which is linked to serum proteins in this esterified form (Szego 1953) The formation of the estroprotein may be part of the mechanism of the activation of estrogen by the liver (Szego and Roberts 1953) Cortisol 4 C^{14} and corticosterone 4 C^{14} incubated in the presence of surviving liver in homologous rat serum were also bound to serum proteins The presence of these steroids inhibited the association of estrone and its metabolites with albumin when the system was incubated with rat liver tissue slices in homologous serum (Szego and Roberts 1956 Szego 1957)

Although the suggestion that liver may play an active role in the formation of serum protein complexes with steroid hormones is of great interest the experimental data which have been offered in support of this concept are still inadequate Formation of plasma protein

The characterization of the state of steroid hormones in blood remains a challenge Despite the efforts for a better understanding of the *in vivo* binding of steroid hormones and their metabolites by plasma proteins there is no information regarding the physicochemical state of the endogenous protein steroid complexes Such a characterization will involve the isolation and characterization of both the protein and the steroid The difficulties which are involved in these studies derive from the fact that steroid hormones are in very low concentration in plasma and that the procedures which are employed for the separation and isolation of the protein steroid complexes may interrupt this association Organic solvents probably disrupt this association and cations like Zn++ employed for plasma protein fractionation may compete with the steroids for the binding sites of the proteins occupied normally by the steroids

Administration of exogenous steroid labeled with carbon 14 offers the advantage of easy detection of the radioactivity in the plasma and plasma fractions However it is not clear to date if such studies advance our knowledge towards the understanding of the physiological state of the protein steroid complexes A factor which may be involved in these studies is the saturation problem If the endogenous steroid occupies the binding sites of the protein which transports this steroid then the exogenous steroid will be associated with another protein and most probably with the albumin Data obtained with exogenous steroids must be characterized as such

An interesting attempt has been made recently by Upton *et al* (1959) to find whether or not the distribution of endogenous cortisol and that of cortisol added to plasma *in vitro* are identical While the results are not conclusive probably because of the limitations of the ultracentrifuge as a means of plasma protein fractionation studies of this kind will be necessary in order to elucidate the problem of the relation of the results obtained with exogenous and endogenous steroid hormones The search for more suitable techniques for the physicochemical characterization of the protein steroid complexes in plasma and their physiological significance is of primary importance for the solution of the problems related to steroid binding by plasma proteins

active estradiol by homogenates of rat liver have shown the presence of an enzyme system which metabolizes estradiol 16 C^{14} to several metabolites and also catalyzes the formation of a liver protein bound estradiol metabolite This enzyme system requires for maximal activity the presence of an electron donating substrate triphosphopyridine nucleotide and oxygen The steroid bound to liver proteins was not released from the proteins by procedures which cleave disulfide glucosidic or ester linkages and was devoid of estrogenic activity Other tissues were less effective in binding the radioactive estradiol

The experimental data from these *in vitro* studies indicated (1) that liver metabolizes estrogen and (2) that liver proteins bind strongly the estrogen metabolites It is not as yet clear whether or not the formation of serum protein estrogen complexes is catalyzed by the liver when estrogen is incubated with liver tissue slices *in vitro* in a homologous serum medium

BIOLOGICAL SIGNIFICANCE OF PROTEIN–STEROID BINDING

One of the reasons for the investigation of the mechanism of the transport of steroid hormones and their metabolites in the blood stream is the possible physiological significance which may exist through such a transport mechanism which involves the binding of these hormones by plasma proteins The interaction of steroid hormones with protein macromolecules may lead to a prolonged life of the hormone in circulation through the low rate of excretion of the steroid bound to plasma proteins The equilibrium state between bound and unbound steroid may be part of the control mechanism of hormone activity in blood Suggestions have been made that the unbound portion of the steroid hormone in the blood stream represents the active fraction available to the cellular membrane (Sandberg and Slaunwhite 1959) Contrarily Szego and Roberts (1953) suggested that the active form of circulating estrogen is the bound form The hormone bound to protein is potentially available for physiological activity by dissociation in the cell membrane Convincing evidence however does not exist for these suppositions

The mechanism of the transport of steroid hormones may involve the formation and the dissolution of highly specific complexes under different conditions Whether or not such complexes require the mediation of enzymes remains to be determined

and purity of most steroid hormones we lack such intimate knowl edge of the human plasma proteins The various fractionation pro cedures although yielding some products of very high purity still fail to achieve perfect separation of the proteins In addition it is possible that products of one fractionation procedure may differ in composi tion from those of another because of differences and variability of human plasmas Hence in the interpretation of the binding of steroids to proteins one should keep in mind the influences of the imperfec tions of the various protein fractions

INTERACTION OF PROTEINS WITH SMALL MOLECULES

Reversible Binding

Since the binding of small molecules to protein is largely a physico-chemical problem several useful equations which describe certain properties of reversible binding are introduced here Complete deriva tions are not given The interested reader is referred to a lucid exposition of the subject by Edsall and Wyman (1958)

Consider a molecule of protein P containing n groups or sites capable of attaching a steroid S Assume that the activities of the components are equal to their concentrations and that all the sites are equivalent and independent When $n = 1$ then



$$P + S \rightleftarrows PS \quad (1)$$

and the association constant k is

$$k = \frac{(PS)}{(S)(P)} \quad (2)$$

Let the number of moles of S bound per mole of P be r forming equation 3

$$r = \frac{(PS)}{(PS) + (P)} \quad (3)$$

Combining equations 2 and 3 yields equation 4

$$r = \frac{k(S)}{1 + k(S)} \quad (4)$$

Extension of these equations to n equivalent and independent sites re quires consideration of one site i and then summation over a total of n sites Thus at the site i

$$r = \frac{k\,(S)}{1 + k\,(S)}, \quad (5)$$

Section 2

The Binding of Estrogens, Androgens and Progesterone by Plasma Proteins *in Vitro**

W Roy Slaunwhite, Jr

INTRODUCTION

In a discussion on the binding of steroids to proteins it is best to define the terms By the term reversible binding is meant a combination of a steroid with a protein molecule in which association or dissociation occurs spontaneously and rapidly The term chemically bound is reserved for a combination of steroid and protein requiring hydrolytic procedures for dissociation and by unbound is meant a state of dissociation The term free as applied to dissociation of steroids and proteins is avoided as it is also commonly used in reference to lack of conjugation of steroids with acids

Steroids occur in two important forms unconjugated and conjugated with either sulfuric or glucuronic acid to form water soluble compounds Although steroids are sometimes spoken of as being conjugated with protein such usage would be confusing in the following discussion and the terminology of the preceding paragraph is employed exclusively At physiological pH the unconjugated steroids are uncharged whereas the conjugated steroids are negatively charged

The source of energy for the binding of organic ions neutral molecules and proteins to protein is still incompletely understood Although it is obvious that ions must have a coulombic interaction due to the attraction of opposite charges it is equally obvious from experimental results that the hydrocarbon portion of the molecule plays an important role Waugh (1954) in a review of the subject postulated that van der Waals forces are minor and attributes the high interaction energy to the formation of new water hydrogen bonds by the water molecules which are displaced from the protein surface upon the coalescence of nonpolar groups The energy of hydrocarbon chain association is believed to be nearly equal to the heat of vaporization of the hydrocarbon namely 1 18 kilocalories per mole of CH_2 groups

Although we know much about the structure physical properties

I am indebted to Dr Alfred Nisonoff and Dr Avery A Sandberg of the Roswell Park Memorial Institute Buffalo New York for their helpful suggestions and comments in the preparation of this section

may be computed where ΔF° is the free energy of association referred to the standard state R the gas constant is equal to 1 987 calories per degree per mole and T is the temperature in degrees Kelvin

If equilibrium constants have been measured at two temperatures T_1 and T_2 the change in enthalpy or heat content ΔH° of the reaction with all components in the standard state may be computed by means of van t Hoff s equation

$$\ln \frac{k_2}{k_1} = \frac{\Delta H}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right) \tag{9}$$

And finally the entropy change ΔS° may be calculated from the relation

$$\Delta F^\circ = \Delta H - T\Delta S^\circ \tag{10}$$

Equilibrium Dialysis

Experimentally there are many ways to determine binding (see Edsall and Wyman 1958) Equilibrium dialysis has probably been used more than any other technique hence it will be described in some detail

Equilibrium dialysis as the name implies is dialysis continued until equilibrium is attained This is best explained by reference to *Figure 7 A suitable semipermeable membrane is employed* Water inorganic ions and low molecular weight organic molecules either charged or neutral may pass freely through such a membrane but

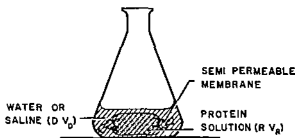


FIGURE 7 The method of equilibrium dialysis R is the amount of ligand inside the semipermeable membrane at equilibrium and D is the amount outside both measured in any units proportional to the amount present V_R and V_D are the corresponding volumes A typical example follows Two milliliters of plasma diluted to 10 ml with saline was placed inside the membrane (V) and 30 ml of saline containing cortisol 4 C14 (2 100 counts per minute) was placed outside (V_D) At equilibrium there were 580 counts per minute in the dialyzate (D) and 1470 counts per minute in the dialyzand (R) The % *unbound* = (580 × 10)/(1470 × 30)100 = 13 the % bound = 100 − 13 = 87

where k is the intrinsic association constant for the formation of PS from P and S, and r is the fraction of site i which is occupied. The total number of occupied sites would be

$$r = nr \tag{6}$$

and since all sites are equivalent,

$$r = \frac{nk(S)}{1 + k(S)} \tag{7}$$

There are several ways to plot this equation but the best is probably the form

$$\frac{r}{(S)} = k(n - r) \tag{7a}$$

Then a plot of $r/(S)$ as ordinate against r as abscissa gives a straight line with an intercept on the abscissa of n and on the ordinate of kn. The sites are then said to be homogeneous.

Frequently the equilibrium constant k is not quite constant but varies about an average value k_0 in a manner which can be described in terms of a Gaussian distribution. The sites are then said to be heterogeneous. Karush and Sonenberg (1949) have derived an equation which describes the relationship of r to k_0 and σ, the index of heterogeneity. Sips (1948) equation, which describes the absorption of gases on solids, has been modified by Nisonoff and Pressman (1958a, b) to describe the binding of small molecules to proteins. Equation 7a then becomes

$$\frac{r}{(S)^{\sigma}} = k_0^{\sigma}(n - r) \tag{7b}$$

Experimentally it can be seen from equation 7b that k_0 is equal to the reciprocal of the unbound steroid concentration when the protein is one half saturated, that is, when $r = n/2$. Although equation 7b does not exactly describe a Gaussian distribution, it fits the data well and it is easier to apply than Karush's equation. The calculation of two parameters, k_0 and σ, although more tedious, affords a more precise evaluation of the interaction under investigation.

In order to be able to compare equilibrium constants obtained at different temperatures, the thermodynamic function

$$\Delta F° = -2.30RT \log k \tag{8}$$

may be computed where ΔF° is the free energy of association referred to the standard state R the gas constant is equal to 1 987 calories per degree per mole and T is the temperature in degrees Kelvin

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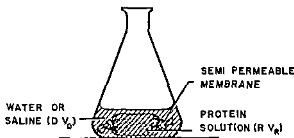


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compounds with a molecular weight greater than 5 000 to 10 000 (proteins glycoproteins polysaccharides etc) can not This method is limited then to the binding of low molecular weight solutes (ligands) to high molecular weight solutes

Assume now an equal concentration of steroid on both sides of the membrane When protein is introduced inside the casing the steroid may bind to it according to equation 1 The concentration of unbound steroid S then being less than that outside the casing steroid diffuses in until equilibrium is restored (The whole process is of course dynamic with association dissociation and diffusion both in and out going on constantly At equilibrium the rates of any pair of reactions such as association dissociation are equal and opposite If equilibrium does not exist one rate becomes larger than the other until equilibrium is restored) All of the steroid outside the casing is obviously unbound Let its concentration be C_U then

$$C_U = \frac{D}{V_D} \tag{11}$$

where D is the total amount of steroid in volume V_D Inside the casing let the total concentration of steroid be C_T and the concentration of bound steroid be C_B then

$$C_T = C_U + C_B = \frac{R}{V_R} \tag{12}$$

where R is the total amount of steroid in volume V_r The percentage of the steroid inside the casing which is unbound will be

$$\%\text{ unbound} = \frac{C_U}{C_U + C_B} \times 100 = \frac{D}{R} \times \frac{V_R}{V_D} \times 100 \tag{13}$$

and the $$\%\text{ bound} = 100\left(1 - \frac{D}{R} \times \frac{V_R}{V_D}\right) \tag{14}$$

In practice the nondialyzable component is usually placed inside the semipermeable membrane or casing in a volume V_R of water or salt solution The same solvent of volume V_D is placed outside The dialyzable solute or ligand may be placed in either compartment The container is then rocked slowly to and fro or moved in such fashion as to ensure continuous circulation of the outer phase The inner phase if desired may be stirred by means of a small glass bead which will move from one end of the casing to the other as the casing is rocked After attainment of equilibrium each phase is analyzed separately for its content of ligand

The diameter of the casing is not critical but it is obvious that equilibrium will be reached quicker if a large surface to volume ratio (small diameter tubing) is employed In practice casing of ¼ to ¾ inch diameter is commonly employed

The volume of solvent outside the casing V_D is usually called the dialyzate That inside the casing V_R has no generally accepted name it is designated here as the dialyzand The size of these volumes is a matter of convenience it is their ratio that is important Depending on the amount of binding the volume ratio must be adjusted so that there is sufficient ligand in each phase at equilibrium for an accurate analysis Sometimes one sees an analysis of only one phase the other being found by difference This practice is always dangerous and sometimes invalid To be valid the absorption of the ligand to the dialysis membrane and/or to the walls of the vessel and the loss in the analytical procedure if any must be corrected for If these conditions are met one may assign the balance of the ligand to the other phase The procedure is hazardous as any error in one phase will produce twice that error in the ratio of D/R Analysis of both phases is preferred Experiments in which there is poor recovery of material should be discarded

The ligand may be placed initially in either compartment Since it is customary to dialyze from the inside out the reverse procedure is called dialysis in the reverse direction or in laboratory jargon reverse dialysis The best procedure is to do both Not only does this afford duplicates but it also yields information regarding the attainment of equilibrium If they differ with the value for dialysis in the reverse direction being lower than the other then dialysis should have been continued longer

ESTROGENS

The work on the *in vivo* binding of estrogen reported by Rakoff *et al* (1943) and Szego and Roberts (1946) has been reviewed by Antoniades in the preceding section of this chapter Bischoff *et al* (1954) challenged the conclusion of Szego and Roberts that estrogens are bound to serum proteins The argument here is purely semantic for the former workers define an estrogen bound to protein as one held to protein by an atomic linkage as the iodine in thyroxin is bound to the phenol group of thyroxin by atomic linkage or by a strong secondary valence as in a conjugated protein

They treat the dispersion of steroids by albumin as a distribution following the classic laws of distribution based on solubility While this is theoretically admissible it is not consonant with general prac tice *in the large field of protein binding* of which steroid protein interactions are a minor part Szego and Roberts have never defined their concept of binding Since in their early reports they speak of equilibrium disassociation and law of mass action it is obvious that they are thinking of binding as a reversible phenomenon Later in animal experiments Szego (1953) described the essential role of the liver in estroprotein formation which almost certainly implies chemical binding

More recently Bischoff and Stauffer (1957) have concluded that albumin is the *most important element in the transport of estrogen* in the serum This important conclusion is based on the fact that estrogens either exogenous or endogenous migrate during dialysis from serum to albumin at the same rate as from albumin to albumin Further evidence is cited in a subsequent paper (Bischoff *et al* 1958)

Boettiger (1946) was the first to measure quantitatively the binding of estrogen to human plasma proteins By means of the equilibrium dialysis technique he found that estriol was 97 per cent bound at estriol concentrations of 0 34 to $2\,0 \times 10^{-4}$ moles per liter

TESTOSTERONE

Most of the investigations of testosterone albumin interaction have employed bovine serum albumin (for further references see Sandberg *et al* 1957) Macek *et al* (1952) determined the solubility of testos terone in human serum albumin Since these investigators used an equilibration time of only 1 hour it is unlikely that they attained equilibrium Also the authors do not state whether they corrected for the partial specific volume and the hydration of the albumin *which is imperative at the high protein concentration* employed

Oyakawa and Levedahl (1958) have concluded that testosterone probably binds to the phenolic hydroxyl of the tyrosyl moieties in albumin This conclusion is based on the fact that while iodination of tyrosyl groups or acetylation of ε amino groups does not influence the binding of testosterone to albumin ketenization produces a decrease in binding which is roughly proportional to the number of hydroxyl groups blocked and which is reversible by hydrolysis of the acetate groups at pH 11 They visualize the testosterone molecule as

binding perpendicular to the chain of the protein and continuous and parallel to the ring structure of the tyrosyl side chains The specificity of the steroid structure has not been investigated although the authors feel that binding may be through the 3 keto group since both *testosterone and 17 methyltestosterone* bind similarly

PROGESTERONE

Westphal *et al* (1955 1956) purported to show that labeled progesterone moved with serum albumin during paper electrophoresis and paper chromatography of albumin or serum Review of the figures however revealed either a fairly constant level of radioactivity which did not vary at all with albumin concentration or a high peak of radioactivity located at or near the point of application As the authors themselves point out the paper competes with the protein for the steroid Daughaday (1958b) has published a method which overcomes this difficulty After dialyzing the protein solution against the electro phoretic buffer to which labeled steroid has been added the buffer is used to saturate the paper Thus the affinity of the paper for a steroid is saturated and the paper no longer competes with the pro tein for the protein bound steroid The disadvantage of this method is the high radioactive background which is necessarily produced One wonders if saturation of the paper with buffer containing non radioactive steroid would be feasible If the adsorption of steroid to the paper were irreversible or if the equilibrium constant for steroid paper association were much larger than that for steroid protein association such a modification would be desirable

More convincing evidence of binding was obtained by Westphal (1955) using free electrophoresis and ultracentrifugation techniques By ultracentrifugation progesterone was 98 4 per cent bound to 4 per cent albumin at 20°C whereas by electrophoresis a value of 98 9 per cent bound to 1 3 per cent albumin was obtained (The total steroid concentration was $2\,1 \times 10^{-6}$ and $4\,1 \times 10^{-6}$ moles per liter respectively) The latter value is undoubtedly erroneous since a lower concentration of albumin should result in less binding The former value agrees well with values obtained by equilibrium dialysis and ultrafiltration

Westphal (1957) and Westphal and Ashley (1958) have also demon strated quantitatively steroid protein interactions by the spectro photometric method Interaction of α β unsaturated 3 ketosteroids

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FIGURE 8 The binding of selected radioactive steroids at 5 C with human plasma protein fractions dissolved in 0.15 M NaCl at pH 7.0 The total steroid concentration was approximately 10^{-7} moles per liter The concentrations of protein are given in grams per hundred milliliters The lower set of values for Fraction III 0 represents the binding of native lipoprotein the higher set of the denatured lipoprotein (From Sandberg, *et al* 1957)

with albumin caused a depression of the molecular extinction coeffi cients at 240 millimicrons which was proportional to the extent of binding This observation strongly suggests that the interaction of progesterone and similar hormones involves the Δ^4 3 keto group Introduction of hydroxy or keto groups into the steroids produced a decrease in the interaction with albumin whereas introduction of a methyl group at carbon 2 strengthened the binding The magnitude of these effects depended on the position and the steric arrangement of the groups introduced into the steroid molecule

COMPARATIVE BINDING OF STEROIDS

Sandberg *et al* (1957) have published a comprehensive treatise on the subject of steroid protein interactions involving a systematic study of all the major protein fractions and the four major classes of steroid hormones both *in vitro* and *in vivo* Radioactive tracers were used in this study for several reasons (1) ease simplicity and rapidity of operation (2) the use of extremely low concentrations of steroid and (3) high sensitivity and accuracy of measurement Dialyses were con tinued to equilibrium usually from both directions Ultrafiltrations were sometimes performed also

In corroboration of Bischoff *et al* (1954) estrone C^{14} either added *in vitro* or injected intravenously could be recovered from blood by extraction with acetone ethanol or chloroform and ethanol Little if any radioactivity was released by alkaline hydrolysis of the pre cipitated proteins

The binding of C^{14}-estradiol 17β and of estrone C^{14} as well as a number of other radioactive steroids is shown in Figure 8 This graph compares the ability of various plasma protein fractions to bind steroids Two corticosteroids are included for comparison Estrone and estradiol are bound most extensively by albumin appre ciably by Fractions IV 1 and IV 4 and least by Fractions I II and III 0 Notice however the pronounced increase in binding produced by denaturation of Fraction III 0 Progesterone and testosterone are bound a little less firmly than the estrogens in most cases In freshly prepared Fraction III 0 however these two steroids bind more strongly than the estrogens

The concentration of protein in Figure 3 expressed in grams per hundred milliliters was made approximately equal to that in plasma Hence one would expect albumin to be responsible for most of the

An attempt was made by Sandberg *et al* (1957) to determine the number of sites on an albumin molecule which will bind certain steroids (Fig 9) Due to the insolubility of estrone progesterone and

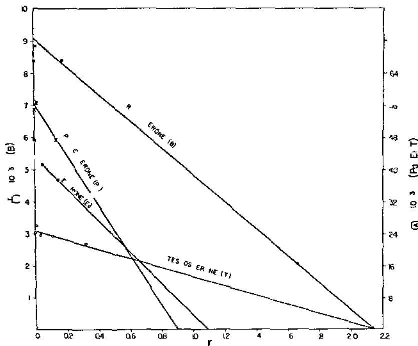


Figure 9 *The binding of selected radioactive steroids in the presence of* increasing amounts of carrier steroid at 5 C to 1 per cent human serum albumin dissolved in 0 16 M NaCl at pH 7 0 Extrapolation of the curves for *estrone progesterone and testosterone yields only minimum values of n* (From Sandberg *et al* 1957)

testosterone in water a wide range of concentration of steroid could not be employed Hence the extrapolation of the experimental points is unduly long and the only conclusion is that there is at least one site on the albumin molecule which binds estrone and progesterone and *two sites which* bind testosterone There may be additional sites possessing lower equilibrium constants

The question as *to whether more than one type of steroid molecule* may bind at any given site has not been adequately investigated Preliminary experiments in which tracer amounts of one steroid competed with large amounts of a second nonradioactive steroid showed the binding of the radioactive steroid to be unaffected by the presence of the second steroid Tested in this way were corticosterone *vs* estrone

transport of estrone and estradiol in the plasma Slaunwhite and Sandberg (1959) have demonstrated that this is indeed the case by the following procedure Plasma diluted 1 5 was placed inside a dialysis bag and 1 gm per hundred milliliters serum albumin was placed outside thus the concentration of albumin was essentially the same on both sides of the membrane If all the binding of plasma is due to its content of albumin estrone C^{14} or estradiol C^{14} placed in either compartment should at equilibrium attain the same con centration inside and outside the dialysis bag If there is an additional component in the plasma binding estrogen the concentration inside the casing will be greater than outside Experimentally the former was found to be true Bischoff and Stauffer (1957) and Bischoff *et al* (1958) have also reached the same conclusion

Conclusions regarding the *strength* of binding cannot be drawn from Figure 8 due to the large differences in molecular weight of the proteins Calculation of equilibrium constants (Equation 2) of binding reveals that for a given steroid all the fractions except Fraction II possess nearly the same strength of binding Equilibrium constants for Fraction II are approximately one order of magnitude lower than for the other fractions For a given protein fraction the greatest energy of binding is usually exhibited by estrone or estradiol but progester one and testosterone are bound most strongly to Fraction III 0 and corticosterone and cortisol to Fraction IV-4

The forces involved in binding are relatively weak For example thermal energy at 5 C is approximately 0 7 kilocalorie per mole On the other hand the equilibrium constant for the association of cortisol and transcortin is $3\ 0 \times 10^7$ liters per mole (Slaunwhite and Sandberg 1959) and of thyroxine and thyroxine binding protein $7\ 9 \times 10^9$ liters per mole (Robbins and Rall 1957) whereas that of estrone and albumin is $4\ 4 \times 10^4$ liters per mole (5 9 kilocalories per mole)

Determinations of binding to albumin performed at 5°C and 25 C and a few determinations performed at 37 C revealed that increasing temperature produced only a small decrease in binding (Slaunwhite *et al* 1959) This means that the enthalpy of binding is small and positive and that most of the energy change is derived from an increase in entropy This effect which is also observed in ion albumin interactions has been attributed by Klotz (1949) to a displacement of hydration water from the ionic groups during binding of dye anions Karush (1950) has proposed that an alteration in the structure of the protein produces the observed entropy change

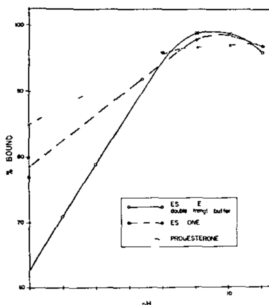


FIGURE 10 The effect of pH and ionic strength on the binding at 5 C of estrone 16 C^{14} and progesterone 4 C^{14} to 1 per cent human serum albumin dissolved in acetate phosphate or glycine buffers (From Sandberg *et al* 1957)

tioned are completely bound in undiluted plasma Corticosterone and cortisol are firmly bound by Fraction IV 4 however this fraction contains only 10 per cent of the total plasma protein whereas Fraction V contains 51 per cent These two fractions cannot therefore account for all the increase As we know the increase is attributable to transcortin the corticosteroid binding protein (see Section III)

The binding of conjugates of steroids to plasma proteins remains mostly unexplored due to the complete absence of labeled conjugates and the scarcity of unlabeled conjugates Through the kindness of Dr John J Schneider who provided us with etiocholanolone glucosiduronate and androsterone sulfate an attempt was made to determine the extent of binding to albumin No consistent quantitative data could be obtained due to the low concentration of steroid remaining in the dialyzate and to the presence of interfering chromogens in the acid hydrolized extract of the interior of the dialysis casing The indication was that *these two conjugates are bound more extensively to* albumin than the corresponding unconjugated steroids

An expedient employed by Slaunwhite and Sandberg (1958) was the use of radioactive urinary conjugates excreted after administration of

C^{14} testosterone C^{14} and progesterone C^{14} and progesterone *vs* estrone C^{14} testosterone C^{14} and corticosterone C^{14} To be definitive this experiment must be repeated using greater amounts of the radioactive steroids

The fact that albumin binds steroids enhances the solubility of the latter in aqueous solutions (Table II) This effect is more pronounced

TABLF II Solubility of Steroids in 1 1er Cent Human Serum Albumin and in 0 16 M NaCl at pH 7 0 and ȷ C

Steroid	Sol bility				D p ing pow	
	N Cl (A)		1% HSA (B)		B − A	
	μg /ml	m l /l te 10^6	μg /ml	m l /lit 10^6	μg /ml	m l /lit 10^6
Est e	1 6	5 9	7 6	27 9	6 0	22 0
Estradiol	2 7	9 9	30 0	110	27 3	100 1
P ogest ro e	3 6	11 4	14 3	45 5	10 7	34 1
Te tost o e	16 1	45 8	39 4	137	23 3	91 2
Corticosteron	118	342	247	741	129	372
Cortisol	197	544	220	607	23	63

if there are multiple sites (compare testosterone and corticosterone with estrone and progesterone) West *et al* (1951) found that 2 31 mg of testosterone could be dissolved in 1 ml of 25 per cent albumin In this way large amounts of steroid could be administered intravenously Rothchild (1952) has also taken advantage of the dispersing power of albumin to prepare solutions with high concentrations of estradiol or testosterone for intravenous administration

In considering binding pH is an important factor The binding of estrone and progesterone to 1 gm per hundred milliliters of albumin was studied in the pH range of 4 to 11 The results shown in Figure 10 reveal an increase in binding with increasing pH for both steroids Above pH 8 however the two steroids differ The binding of progesterone increases very slightly with a further increase of pH but that of estrone reaches a maximum between pH 9 and pH 10 and then decreases at pH 11 The fact that estrone is a weak acid pK = 9 36 ionizing in the pH range of 9 to 10 may be responsible for this difference

From our knowledge of the composition of plasma one should be able to predict roughly how much of each steroid would be bound when added to plasma *in vitro* Estrone estradiol progesterone and testosterone were bound as predicted but corticosterone and cortisol which are more weakly bound to albumin than the first four men

TABLE IV The Binding of Testosterone to Bovine Serum Albumin

Bo ine rum album	T	pH		$K \times 10^{-4} M^{-1}$	Ref enc
U mod f d	25	7 4	5 0	2 97	*Le edahl (1955)*
U od f d	37	7 4	9 3	1 98	Le edahl and B n stein (1954)
Ac tylat d (47 5%)	37°	7 4	10 5	1 97	Levedahl and B t (1954)
Ac tyl t d (91)	37	7 4	9 7	2 06	Le dahl d B nst in (1954)
U dif d	*25*	*7 4*	—	*2 26*	*Le dahl a d B nste (1954)*
36 At m d ne	25°	7 4	—	2 66	*Le dahl and Be n te (1954)*
135 At m odine	25°	7 4	—	1 73	Le edahl a d Be nste (1954)
U m d f d	37°	7 4	—	1 46	Lev dahl d Be n te (1954)
39 Atom i d	37°	7 4	—	1 66	Le dahl and B n te (1954)
U od f d	25°	9 2	—	3 20	Le edahl d Be te (1954)
38 Atom e	*25°*	*9 2*	—	*4 01*	*Le edahl and B nst (1954)*
Ket d (75)	25	7 4	—	0 46	Le ed hl d B ste (1954)
Kete ed d hyd ly (56%)	25°	7 4	—	0 72	Le dahl d B nst (1954)
K t d (41%)	25	7 4	—	1 12	Le edahl d Be nst (1954)
K t i ed d h d oly (32)	25	7 4	—	1 27	Le dahl d B nste (1954)
Ket z d (35%)	*25°*	*7 4*	—	*1 61*	*Le dahl and Be te (1954)*
Ket d a d hydr ly (25)	25	7 4	—	1 85	Le dahl and B te (1954)
U modif d	25	7 4	—	2 26	Le dahl d Be n t (1954)
U dif d	37	2 3	167	0 59	L dahl and P lm tt r (1956)
U m d f d	37	3 4	10	0 93	Le dahl d P lm tte (1956)
U m dif d	*37*	*4 2*	*10*	*1 93*	*Le dahl and P lm tte (1956)*
U m d f d	37	5 6	16	1 55	Le dahl and P lm tte (1956)
U mod f ed	37	6 2	7 0	1 67	Le dahl and Pe lm tte (1956)
U mod f d	37°	7 1	6 7	2 00	Le dahl and P lm tte (1956)
U m difi d	37	7 7	6 2	2 25	Le dahl and P lm tt (1956)
U m dif d	37	8 7	1 1	4 16	Le dahl and P lm tt (1956)
U m dified	37°	9 0	5 9	3 14	L dahl and P lm tt (1956)
U m dif d	*37*	*9 7*	*5 0*	*3 91*	*Le dahl and P lm tte (19 6)*
U m dif d	37	11 6	5 0	0 39	Le dahl and P lm tte (1956)
U mod f d	37	11 7	8 0	0 29	Le dahl nd Pe lm tt (1956)
U m dif d	37	12 3	0 6	0 32	Le dahl and P lm tte (1956)
U m dif d	25	6 9	4 0	2 37	Le edahl and P lm tt (1956)
U dif d	25°	7 5	4 0	2 84	Le dahl and P lm tt (1956)
U m dif d	25	8 3	5 5	3 79	Le dahl and P lm tte (1956)
U modif d	25	9 3	4 6	4 59	Le dahl nd P lm tte (1956)
U m d f d	25	9 7	4 0	4 21	Le d hl a d P lm tt (1956)
U m dif d	25	10 2	5 0	5 35	Le dahl d P lm tte (1956)
U d f d	25	10 4	5 3	5 51	Le dahl and P lm tt (1956)
U dif d	25	10 7	6 4	5 74	Le d hl and P lm tt (1956)
U modif d	25	11 0	4 0	6 09	Le dahl and P lm tt (1956)
U m dif d	25	11 2	4 8	5 62	Le dahl nd P lm tte (1956)
U m d f d	25	11 4	5 0	3 60	Lev dahl and P lm tte (1956)
U m dif d	25	11 5	12 3	1 75	Le dahl and P lm tter (1956)
U m dif d	25	11 6	5 8	0 67	Le dahl d P lm tte (1956)
U odifi d	25	11 8	3 6	1 32	Le dahl and P lm tt (1956)
U dif d	25	12 0	5 0	1 68	Le dahl and P lm tte (1956)
U m d fi d A	25	7 4	10	1 9	Sch llma et l (1954)
U odif d B	25	7 4	6	2 3	Sch llma t al (1954)
U odifi d C	25	7 4	5	4 0	Sch llman t l (1954)
U mod f d B	8	7 4	7	1 7	Sch llman et l (1954)
U odif d B	37	7 4	4	2 0	Schellm t l (1954)

TABLE III The Binding of Various Steroids to Bovine Serum Albumin (BSA)

St d	25						37 5						R f
	N Cl b ff			BSA			N Cl b ff			BSA			
	C	pH	S†	C	pH	S†	C	pH	S†	C	pH	S†	
Estron							155	6 5 8 1	5	3 1	5 3	59	Bischoff and Katherman (1952)
E t e palmitate										3 1	8 5 8 8	160	Bischoff and Katherman (1952)
Est ad ol										3 1	7 5	<7	Bischoff and Kath rman (1952)
E tradiol	—	7 3	0 6	5	7 3	310							Eik Nes et al. (1954)
	155	8 5	6 6	3	5 5	82	155	7 0	9 3	1	5 5	71	Bi ch ff and Katherman (1948)
							155	8 6	8 7	3	5 5	180	Bi choff and Katherman (1948)
E tradiol										3 1	8 5	490	Bischoff a d Katherman (1952)
Estrad ol b nz te										2 9	5 3	6	B choff et al (1952)
Testo tero	0		95	3	5 3	990	0	—	126	1	5 4	520	B ch ff and Pilhorn (1948)
Te tost	172	—	78	3	7 2	1250	172	—	113	3	5 3	1160	B schoff and Pilhorn (1948)
							—	7 3	125	5	7 3	2150	Eik Nes et al. (1954)
Te toster e										1	5 4	530	Bi choff a d Stauffer (1954)
T stoste e propi te										1	7 4	670	Bischoff and Stauffe (1954)
							0	—	11	2 9	5 3	400	Bisch ff et al. (1952)
T sto teron palmitate										2 9	8 3	620	B s hoff et al (1952)
										3 2	7 7	21	B schoff et al. (1952)
Methylt t t e							—	7 3	120	5	7 3	1750	Eik Nes et al. (1954,
Andro ta l	—	7 3	0 2							5	7 3	140	Eik Nes et al (1954)
Andro ta 3 17 d ne	—	7 3	220							5	7 3	3200	Eik Nes et al. (1954)
Δ^4 Andro te 3 17-d e	—	7 3	200							5	7 3	2500	Eik Nes et al. (1954)
Δ^4 Androste 3 one	—	7 3	1	5	7 3	490							Eik Ne et al (1954)
Andr te o				5	7 3	1350	—	7 3	43				Eik Nes et al. (1954)
Dehydro so droste on	—	7 3	70				—	7 3	100	5	7 3	2850	Eik Nes et al (1954)
Etioch l ol e				5	7 3	900							Eik N s et al. (1954)
P g st e				3	5 3	23	0	—	42	1	5 3	14	Bis hoff and Pilhorn (1948)
				3	7 1	30	172	—	36	3	5 3	32	Bisch ff and PUh n (1948)
										3	7 4	41	Bischoff and Pilhorn (1948)
										3	8 1	65	Bi choff and Pilhorn (1948)
Desoxyco ticostero							—	7 3	180	5	7 3	1030	Eik Nes et al (1954)
Co ti o							—	7 3	600	5	7 3	1400	Eik Ne et al. (1954)
Chol sterol	—	7 3	1	5	7 3	1							

C m l co u f N Cl b ff th t ti f BSA l gram p t
†S th l bili y f te d exp es ed in ml m les p lit

Section 3

The Binding of Corticosteroids by Serum Proteins *in Vitro*

William H Daughaday

Until recently the measurement of the concentration of a hormone in plasma has been considered the ultimate criterion for estimating effective hormonal levels The realization that certain hormones especially those of the thyroid and adrenal glands and the gonads circulate in the blood bound to serum protein makes information concerning the activity (used in its physicochemical sense) of a given hormone in blood of great importance in evaluating physiological events It is probable that the small concentration of unbound hormone of plasma is the fraction of total circulating hormone that determines the diffusion across capillary and cellular membranes and is the moiety which is under pituitary regulation (Robbins and Rall 1957)

THE BINDING OF CORTICOSTEROIDS BY ALBUMIN

The availability of albumin preparations of high purity and the demonstrated ability of albumin to bind many ionic and non ionic substances of diverse properties have prompted the investigation of the binding of corticosteroids by this plasma protein Eik Nes *et al* (1954) compared the solubility of cortisone and other steroids in buffer solution with that observed in solutions of bovine serum albumin The enhanced solubility in the protein solution of the twelve steroids studied was inversely proportional to the degree of water solubility and the number of polar groups Cortisone and desoxycorticosterone were soluble to a lesser extent than estradiol testosterone and a number of related steroids The fact that bovine serum albumin binds cortisone relatively weakly as compared to other steroids was also established by dialysis equilibrium and partition analysis (Schellman *et al* 1954)

When examined at relatively high concentrations of steroid (Daughaday 1956b) binding of corticosteroids by human serum albumin and bovine serum albumin was found to be of comparable extent With concentrations of unbound cortisol of less than 65 micromoles per liter the binding by human albumin (4 per cent solution) was greater

labeled hormones The urine was used as excreted which means t the results were an average of the binding of the various metabol conjugated in two or more ways The results are therefore of limi usefulness but they do show that steroid conjugates especially th of estrone estradiol testosterone and progesterone bind avidly albumin

The material presented in this section refers primarily to the bi ing of steroids to human plasma proteins *in vitro* Many valua experiments using bovine serum fractions have been reported by v: ous investigators The results of those experiments in which quant: tive measurements of the binding of steroids to bovine serum albun were made are shown in Tables III and IV In Table IV determinati of *n* the number of sites is less accurate than *nk* and hence cc parisons should be made using the latter The data of Table III ag: show the lack of specificity of albumin Of the nineteen steroids a steroid derivatives shown only four — cholesterol progesterone a the palmitates of estrone and testosterone — failed to be markedly mc soluble in BSA than in water

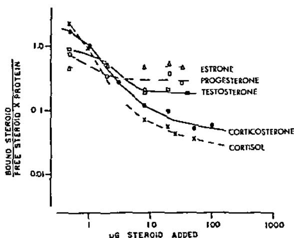


FIGURE 11 Equilibrium dialysis of human serum with estrone 16 C^{14} pro gesterone 4 C^{14} testosterone 4-C^{14} and cortisol 4 C^{14} The micrograms of The ordinate is an index of the affinity of binding (From Daughaday 1958a) steroid added per 10 ml of serum are plotted logarithmically on the ab cissa

protein Increasing the added cortisol to 10 μg per 10 ml of plasma reduced the binding to between 64 and 69 per cent The results of many such experiments indicate that the cortisol of the plasma is vir tually completely (99 per cent) bound to protein at concentrations be low 20 μg per 100 ml of plasma At higher concentrations of cortisol the proportion bound to protein falls considerably (Fig 12) Similar observations have been made independently in two other laboratories (Bush 1957 Slaunwhite and Sandberg 1959)

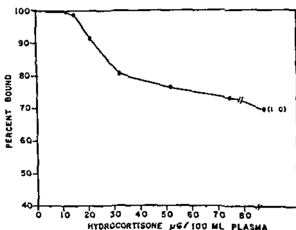


FIGURE 12 Changes in the percentage of cortisol bound by human plasma as a function of total cortisol concentration (From Daughaday 1958a)

than that by bovine albumin Westphal (1957) has presented spectrophotometric evidence that human albumin interacts more firmly with the Δ^4 3 keto group of steroid hormones than does bovine albumin He found that a decrease in the characteristic ultraviolet absorption of the steroid occurred when it was bound to protein The decrease with human albumin was greater than with bovine serum albumin

The introduction of corticosteroids labeled with carbon 14 as research tools has facilitated measurement of the binding of steroid hormones by albumin Sandberg *et al* (1957) using both ultrafiltration and dialysis equilibrium measurements found that the equilibrium constant of binding by human albumin of cortisol 4 C^{14} and cortisone 4 C^{14} was $0\ 5 \times 10^4$ this being less than that observed with other steroid hormones When the number of moles of steroid per mole of albumin (*r*) was plotted against *r*/[*s*] where [*s*] is the unbound steroid concentration) evidence for two binding sites for corticosterone was obtained Moreover the binding sites on the albumin molecule for the various steroid hormones are probably not identical For instance saturation of the system with progesterone does not inhibit the binding of corticosterone 4 C^{14}

CORTICOSTEROID-BINDING GLOBULIN

The relatively weak binding of corticosteroids by albumin led to the general supposition that these steroids were bound less firmly to plasma proteins than androgens progesterone and the estrogens Dialysis equilibrium experiments in which easily measured amounts of cortisol were added to plasma seemed to support this view in that nearly 20 per cent of the steroid in plasma was unbound However when plasma was obtained 2 hours after the administration of cortisone acetate all but 5 per cent of the unconjugated Porter Silber chromogens were protein bound as measured by equilibrium dialysis (Daughaday 1956b) Only 65 per cent of the Porter Silber chromogens conjugated with glucuronic acid were similarly bound

These results prompted a more extensive study of corticosteroid binding by plasma at physiological concentrations of hormones (Daughaday 1958a) The binding of cortisol 4 C^{14} and corticosterone 4 C^{14} by plasma at 4 C was found to be critically dependent on the amount of steroid added to the dialysis equilibrium system (Fig 11) When 0 5 μg of labeled cortisol was added to 10 ml of plasma virtually all of the steroid within the dialysis bag (98 to 99 per cent) became bound to

of a 2 methyl group or an unsaturation at the 1 2 position only slightly reduced the ability to displace cortisol

Parallel experiments conducted with corticosterone-4 C^{14} and human plasma yielded equivalent results Cortisol proved to be just as effective an inhibitor of corticosterone 4 C^{14} binding as corticosterone

The structural features which a steroid must possess for maximal binding by corticosteroid binding globulin differ considerably from those required for maximal binding by albumin In particular hydroxyl groups at the 11β 17α and 21 positions decrease albumin binding but increase the binding by the corticosteroid binding globulin

CORTICOSTEROID BINDING BY PLASMA PROTEIN FRACTIONS

The ability of plasma protein fractions obtained by the cold ethanol procedure of Cohn to bind corticosteroids has been examined to learn the nature of the binding proteins When relatively large amounts of cortisol were added to such fractions (in dialysis equilibrium experiments) the greatest binding was observed in Fraction V (albumin) (Daughaday 1956b) When the experiments were repeated using the small amounts of cortisol 4 C^{14} which had been used in the experiments conducted with plasma quite a different result was obtained (Daughaday 1958a) It was now evident that Fraction IV-4 contained the highest affinity for cortisol The binding of a number of steroid hormones by Fractions IV 4 and V (albumin) has been compared in Figure 13

Sandberg *et al* (1957) using similar methods have also found that Fraction IV 4 had the highest affinity for cortisol although some corticosteroid binding activity was demonstrable in all plasma protein fractions Cortisone was much less bound than cortisol by Fraction IV-4 a finding that is in agreement with the previously discussed structural specificity of binding by the corticosteroid binding globulin

Although corticosteroid binding globulin has been found in Fraction IV-4 the binding capacities of the samples examined by the author are only slightly greater than those of native plasma The failure of the fractionation procedure to concentrate corticosteroid binding globulin indicates either that denaturation has occurred or that corticosteroid binding activity may be present in Fraction VI which thus far has not been adequately examined In any case the alcohol frac

This same phenomenon was observed with corticosterone but was much less evident with testosterone and progesterone The combining affinity of plasma for estrone was very little influenced by the amount of steroid added to the dialysis system within the limits examined (Fig 11)

Recently it has been demonstrated by Slaunwhite and Sandberg (1959) that the binding of cortisol by the corticosteroid binding globulin (transcortin) is greatly influenced by temperature The binding at 37°C was found to be considerably less than at 4°C A similar change in the binding of cortisol by albumin does not occur Caution is necessary therefore in extrapolation of the results obtained with dialyses at 4°C to the conditions existing in the body The ultrafiltration measurements of Mills and Bartter (1959) indicate that roughly 10 per cent of the plasma cortisol is ultrafiltrable at body temperature

These observations on the binding of steroids led to the hypothesis that there is present in serum a very small concentration of binding sites with an affinity which is greatest for the corticosteroid hormones less for testosterone and progesterone and undemonstrable for estrogens When these binding sites of high affinity for corticosteroids are occupied binding occurs to a significant degree on albumin and perhaps other serum proteins The corticosteroid binding sites have been attributed to a protein hereafter designated as the corticosteroid binding globulin

The molecular specificity of the binding site of the corticosterone binding globulin can be studied in whole plasma because the binding affinity of this protein for cortisol is so much greater than that of albumin and other plasma proteins This question has been approached indirectly by measuring the ability of nonradioactive steroids to inhibit the binding of cortisol-4-C^{14} by plasma (Daughaday 1958a) Hydroxylation at the 17α 21 or 11β position increases the ability of a nonradioactive steroid to inhibit binding of cortisol-4-C^{14} The effects of other substitutions on the 11 carbon atom are of interest Both the 11α hydroxyl group and the 11 keto group greatly reduced the ability of a steroid to displace cortisol from the binding site Certain other structural changes also decreased the affinity of a steroid for the cortisol binding site This was found to be true of the 18 aldehyde group (as in aldosterone) and the 9α fluoro group (as in 9α fluorohydrocortisone) and in reduction of the Δ^4 3 keto group (as in tetrahydrocortisol) However modification of cortisol by introduction

for paper electrophoresis (Daughaday 1956c) Plasma containing the C^{14} labeled steroid under study has been dialyzed initially against the buffer which subsequently was used for electrophoresis With this modification the steroid protein complex migrates through a buffer containing an equilibrium concentration of labeled steroid Under these circumstances there is no net dissociation of the steroid from the protein Unequivocal demonstration of the binding of cortisol and corticosterone in plasma with protein having the mobility of albumin at pH 8 8 has been achieved A lesser degree of binding has been observed in experiments with human albumin and very little binding could be demonstrated with bovine serum albumin

Continuous flow paper electrophoresis has been used in subsequent experiments because it allows the electrophoresis of larger quantities of serum and the use of much smaller amounts of labeled steroid (Daughaday 1957) This latter consideration is of great importance in view of the binding characteristics of plasma As in the earlier experiments the buffer was dialyzed against serum containing labeled steroid prior to electrophoresis This same buffer was used to conduct the electrophoresis Figure 14 presents the result of an electrophoresis of human serum and corticosterone-4 C^{14} in barbital buffer pH 8 8 and an ionic strength of 0 045 Virtually all the radioactivity appeared

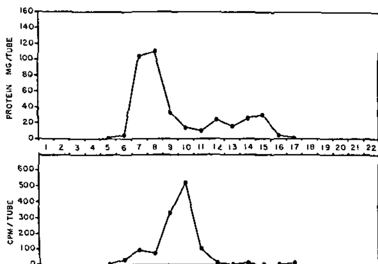


FIGURE 14 Continuous flow paper electrophoresis of human serum and corticosterone 4 C^{14} Barbital buffer pH 8 8 ionic strength 0 045 Serum applied to curtain above tube 14 The numbers one through twenty two between the curves refer to the collection tubes of the Misco apparatus (From Daughaday 1957)

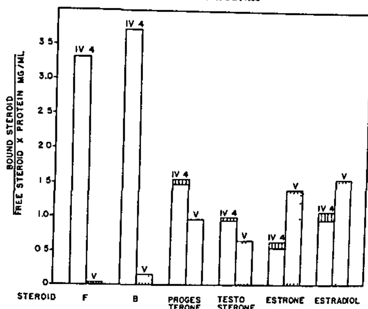


FIGURE 13 The binding of cortisol 4 C^{14} (F) corticosterone 4 C^{14} (B) pro gesterone 4 C^{14} testosterone 4 C^{14} estrone 16 C^{14} and estradiol 16 C^{14} by human plasma fractions V and IV 4 The dialysis equilibrium system con sisted of 5 ml of solutions containing $3\,2 \pm 0\,2$ per cent protein in buffer and 40 ml of buffer containing 0 25 μg of the indicated labeled steroid (From Daughaday 1958a)

tionation procedure seems to have little promise as a method of purification of the corticosteroid binding globulin

IDENTIFICATION OF CORTICOSTEROID-BINDING GLOBULIN BY ELECTROPHORESIS

The successful use of paper electrophoresis in the study of thyroxine binding by serum prompted the application of these methods to the study of corticosteroid binding At first cortisol 4 C^{14} was simply added to plasma and subjected to standard paper electrophoresis As observed by Westphal *et al* (1956) no significant migration of the applied radioactivity occurred The failure to demonstrate protein binding electrophoretically in these experiments is due to the reversi ble character of the steroid protein interaction The protein with its *bound steroid advances through a buffer free of steroid* and under goes continuous dissociation The dissociated steroid is then left behind the migrating protein

Electrophoretic demonstration of corticosteroid binding by serum proteins was accomplished using a modification of the usual procedure

terone-4-C^{14}. However, the electrophoresis of progesterone-4-C^{14} with human serum resulted in two definite peaks of bound radioactivity; the larger of the two peaks occurred in the same position as the corticosteroid-binding globulin, and the smaller of the peaks occurred in the albumin area. This result is consistent with the observed binding of progesterone-4-C^{14} by Fractions IV-4 and V (albumin) shown in Figure 13. The affinity of this steroid for Fraction IV-4 is much less than that of cortisol, but its affinity for albumin is much greater. Saturation of the corticosteroid-binding globulin by the addition of cortisol to the initial dialysis system displaced progesterone-4-C^{14} almost completely from the corticosteroid-binding globulin.

Continuous flow paper electrophoresis of larger amounts of serum has been done at pH 5.2 with the Spinco apparatus. The corticosteroid-binding activity of the eluate fractions has been measured by subsequent dialysis equilibrium and found to correspond exactly to the position of the corticosteroid-binding globulin determined directly when the electrophoresis was carried out in the presence of labeled steroid. Approximately a fiftyfold purification of the corticosteroid-binding globulin has been achieved by this procedure.

PROPERTIES OF CORTICOSTEROID-BINDING GLOBULIN

Despite the fact that corticosteroid-binding globulin has not been isolated in any satisfactory degree of purity, certain estimates concerning the amount of this material and the intensity of the interaction with cortisol can be made similar to those made for thyroxine-binding protein by Robbins and Rall (1957). The apparent saturation of cortisol-binding sites at cortisol levels of 20 μg per hundred milliliters of serum would suggest that the concentration of the binding sites of corticosteroid-binding globulin is approximately 5.5×10^{-7} molar. Assuming that 50,000 represents the equivalent weight for each binding site (Robbins and Rall, 1957), 100 ml. of serum would contain only about 3 mg. of corticosteroid-binding globulin.

The association constant K for the binding of cortisol by corticosteroid-binding globulin can be estimated from the dialysis equilibrium results at 4°C. as follows:

When 0.5 μg of cortisol-4-C^{14} was added to 10 ml. of serum,

$$K = \frac{[CBG \cdot F]}{[CBG][F]} = \frac{(4.1 \times 10^{-7})}{(1.4 \times 10^{-7})(4 \times 10^{-9})} = 0.7 \times 10^{9}$$

to be bound by a protein component which was clearly separable from the albumin peak

Electrophoresis in acetate buffer pH 5 2 has been particularly useful in the study of corticosteroid binding because at this pH the greatest concentration of labeled steroid has been found in tubes which are closer to the anode than those containing albumin and the great mass of serum protein An electrophoresis of serum with corticosterone 4 C^{14} is illustrated in Figure 15 The tubes with the bound radio-

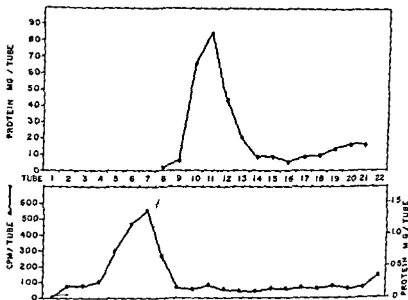


FIGURE 15 Continuous flow paper electrophoresis of human serum and corticosterone 4 C^{14} Acetate buffer pH 5 2 0 02 M Serum was applied to curtain above tube 12 The dotted line in the lower graph is the protein concentration measured by a micromethod (From Daughaday 1958b)

activity contain little protein In other experiments these tubes have been found to contain considerable amounts of bound hexose and bound glucosamine A comparison of the electrophoretic pattern with that obtained by Mehl *et al* (1949) with serum at pH 4 5 suggests that the migration of the corticosteroid binding globulin is intermediate between the component which these authors have called M1 and the slower mucoprotein component M2 Thyroxine binding globulin has the mobility of M2 (Robbins *et al* 1955) which is quite similar to that observed with the corticosteroid binding globulin

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Continuous flow *paper electrophoresis of larger amounts of serum* has been done at pH 5 2 with the Spinco apparatus The corticosteroid binding activity of the eluate fractions has been measured by subsequent dialysis equilibrium and found to correspond exactly to the position of the corticosteroid binding globulin determined directly when the electrophoresis was carried out in the presence of labeled steroid. Approximately a fifty fold purification of the corticosteroid binding globulin has been achieved by this procedure

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The association constant K for the binding of cortisol by corticosteroid binding globulin can be estimated from the dialysis equilibrium results at 4°C as follows

When 0 5 µg of cortisol-4 C^{14} was added to 10 ml of serum

$$K = \frac{[CBG\text{-}F]}{[\text{illegible}]} = \frac{(4\ 1 \times 10^{-7})}{[\text{illegible}]} = 0\ 7 \times 10^{9}$$

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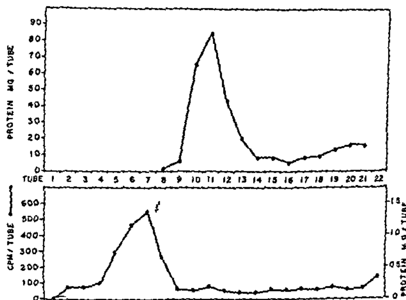


FIGURE 15. Continuous flow paper electrophoresis of human serum and corticosterone-4-C^{14}. Acetate buffer pH 5.2, 0.02 M. Serum was applied to curtain above tube 12. The dotted line in the lower graph is the protein concentration measured by a micromethod. (From Daughaday, 1956b.)

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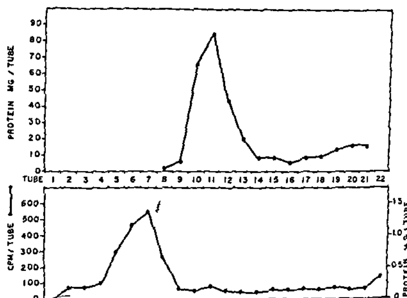


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[*CBG F*] is the molar concentration of the steroid protein complex, essentially equivalent to the initial cortisol of the serum (approximately 1 0 μg), plus the added cortisol (0 5 μg), in 10 ml of serum (4 1 $\times 10^{-7}$ M) The contribution of albumin and other serum proteins to cortisol binding at this steroid concentration can be shown to be insignificant [*CBG*] is the concentration of the corticosteroid binding globulin with unoccupied binding sites This is calculated as the difference between the total concentration (5 5 $\times 10^{-7}$) less *CBG F* [*F*] represents the concentration of unbound cortisol experimentally observed to be about 1 per cent of total cortisol of serum therefore, 4 $\times 10^{-9}$

Estimates of the concentration and binding affinity of corticosteroid binding globulin for cortisol have been compared with similar estimates by Robbins and Rall (1957) for thyroxine binding globulin (Table V) It would appear that the affinity of the corticosteroid binding globulin for cortisol is only one order of magnitude less than that of the thyroxine binding globulin for thyroxine and about four orders of magnitude greater than the association constant for the binding of cortisol by albumin which is given by Sandberg *et al*

TABLE V Comparison of Corticosteroid binding Protein and Thyroxine *binding Protein*

	Corticosteroid-binding protein	Thyroxine binding protein*
Concentration		
Molar	5 5 $\times 10^{-7}$	2 6 $\times 10^{-7}$
Mg /100 ml †	2 75	1 3
Association constant	0 7 $\times 10^{9}$	7 9 $\times 10^{9}$
Electrophoretic mobility		
Alkaline pH	alpha globulin (pH 8 8)	between alpha 1 and alpha 2 (pH 8 6)
Acid pH	between M1 and M2 at pH 5 2	M2 at pH 4 5
Cohn fractionation	IV 4 (? ? VI)	IV 4 (? VI)

*From Robbins and Rall (1957)

†Assuming a molecular weight of 50 000 per binding site

(1957) as $0\ 5 \times 10^4$ The electrophoretic mobility the distribution in fractions of serum obtained by the Cohn method and the ratio between the usual physiological level of the hormone and the total binding capacity of the binding protein are also strikingly similar It is tempting to interpret these similarities as indicative of a more general physiological role for the serum acid mucoproteins

Certain other properties of the corticosteroid binding globulin have been observed (Daughaday and Mariz 1959) At a pH below 5 2 the *corticosteroid binding globulin is irreversibly inactivated in acetate* buffers The material is also denatured within 10 minutes at temperatures between 50 and 60 C Corticosteroid binding globulin is precipitated by 45 to 55 per cent saturated ammonium sulfate at neutral pH and 4 C There is little loss of activity with lyophilization

ALTERATIONS OF ACTIVITY OF CORTICOSTEROID-BINDING GLOBULIN

Preliminary observations have been made of the activity of cortico*steroid binding globulin in the sera in physiological states and in a* number of pathological conditions (Daughaday 1958c) The sera from 8 normal adults contained approximately the same binding activity as measured by dialysis equilibrium About 99 per cent of the cortisol-4 C^{14} was bound to protein and there was no apparent major sex difference Isolated observations on sera of patients with Addison s disease hyperthyroidism hypothyroidism acromegaly and panhypopituitarism failed to show major alterations in corticosteroid binding globulin activity

The alterations of corticosteroid binding by plasma protein which occur in pregnancy and after the administration of large doses of *estrogen are of great interest It has been established that the concen*tration of cortisol in the plasma rises in these conditions without signs of hyperadrenalcorticism Evidence of increased protein binding of cortisol in the plasma of individuals with high estrogen levels was obtained by Mills and Bartter (1959) using ultrafiltration and by Sandberg and Slaunwhite (1959) by dialysis equilibrium. Therefore the changes in plasma cortisol are probably the result of increased binding by plasma proteins without alteration of the unbound cortisol and it is the unbound cortisol which determines the biologic action of the hormone

[*CBG F*] is the molar concentration of the steroid protein complex, essentially equivalent to the initial cortisol of the serum (approximately 1 0 μg), plus the added cortisol (0 5 μg), in 10 ml of serum (4 1 × 10^{-7} M) The contribution of albumin and other serum proteins to cortisol binding at this steroid concentration can be shown to be insignificant [*CBG*] is the concentration of the corticosteroid binding globulin with unoccupied binding sites This is calculated as the difference between the total concentration (5 5 × 10^{-7}) less *CBG F* [*F*] represents the concentration of unbound cortisol, experimentally observed to be about 1 per cent of total cortisol of serum therefore, 4 × 10^{-9}

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†*Assuming a molecular weight of 50 000 per binding site*

cerebral spinal fluid (Daughaday Kozak and Biederman 1959) This fact is important in interpreting the low concentrations of 17 hydroxycorticosteroids which have been reported in spinal fluid (Sandberg *et al* 1954) Probably unbound cortisol in this body fluid is normal whereas the concentration of bound cortisol is much less than in plasma Little binding of cortisol occurs with amniotic fluid The corticosteroid binding activities in a number of transudates and exudates were found to be variable being highest in bloody fluids and the fluids with the highest protein concentration

Preliminary observations indicate that corticosteroid binding globulin is virtually absent from the serum of dogs Rat serum on the other hand appears to contain a higher cortisol binding capacity than human serum (Daughaday 1958c) These species differences should help in assessing the influences of cortisol binding globulin on the dynamics of cortisol transfer across capillary and other cellular membranes

CONCLUSIONS

Dialysis equilibrium experiments with C^{14} labeled steroids have established the presence of a protein (corticosteroid binding globulin) in human serum with a very high affinity for the active corticosteroid hormones cortisol and corticosterone

Information has been obtained concerning the structural specificity of the corticosteroid binding globulin by measuring the ability of related nonradioactive steroid to inhibit the binding of cortisol-4 C^{14} and corticosterone-4 C^{14} The oxygenated functions of the active corticosteroids seem to participate in the binding The type of oxygen function at carbon 11 proved to be of great significance Although a β hydroxyl group enhanced binding both an 11α-hydroxyl and an 11 keto group weakened the protein steroid interaction

The electrophoretic mobility of the corticosteroid binding globulin has been measured using continuous flow paper electrophoresis in which the electrophoretic buffer had been pre-equilibrated with the serum containing the labeled steroid. With cortisol-4 C^{14} and corticosterone-4 C^{14} the radioactivity migrates almost exclusively with a protein having the mobility of an α-globulin in barbital buffer pH 8 8 and as an acid glycoprotein in acetate buffer pH 5 2

The concentration of the corticosteroid binding globulin in normal serum is very small At plasma cortisol concentrations above 20 μg per one hundred milliliters the binding sites are virtually all occupied.

Qualitative as well as quantitative changes in the corticosteroid binding proteins in pregnancy and after estrogen treatment are suggested by the observations of Daughaday and Mariz (1959) A system of double equilibrium dialysis was used which permitted a direct comparison of the degree of binding of cortisol control plasma and of plasma from pregnant women or estrogen treated patients at exactly the same concentration of unbound cortisol No increase in cortisol binding was noted in the estrogen plasma when the comparison was made at 4°C with 0 5 μg of cortisol 4 C^{14} per 10 ml of plasma added to the system If however the comparison was made at 37°C the binding of the estrogen plasma was clearly much greater than that of the control plasma Even at 4°C the greater binding activity of the estrogen plasma could be clearly shown when the amount of cortisol added to the system was increased to 5 5 μg per 10 ml of plasma These paradoxical results suggested to the authors that estrogens lead to the appearance of a binding protein whose affinity for cortisol is not increased at 4°C as much as that of the normal corticosteroid binding globulin The effect of cortisol loading on relative binding suggested that the total binding capacity of the estrogen induced binding protein was considerably larger than that of corticosteroid binding globulin In a pool of plasma from estrogen treated patients studied extensively the estrogen induced binding protein appeared capable of binding about 70 μg of cortisol per 100 ml at 4 C Attempts to separate the two postulated corticosteroid binding proteins by physical means have thus far been unsuccessful

It is interesting to note that in contrast to maternal plasma corticosteroid binding by cord blood plasma has been found to be less than normal by Sandberg and Slaunwhite (1959) and Daughaday Kozak and Biederman (1959) This observation partially explains the fact that the concentration of cortisol in plasma from cord blood is considerably lower than in the mother

Significant decreases in corticosteroid binding activity were observed in the sera of a number of patients exhibiting gross abnormalities of plasma proteins including cirrhosis nephrosis multiple myeloma and monocytic leukemia A particularly striking reduction of the activity of the corticosteroid binding globulin was observed in serum from a patient with subacute hepatic necrosis The concentration of unbound cortisol-4 C^{14} within the dialysis bag was nearly fifteen times normal

Little corticosteroid binding globulin activity has been found in

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PART FIVE

Hormones Produced by Thyroid Gland, Adrenal Medulla and Placenta

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PART FIVE

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CHAPTER XV

*Thyroid Hormones**

Sidney H. Ingbar *and* Norbert Freinkel

THE BIOSYNTHESIS OF THYROID HORMONES
- Intrathyroid metabolism of iodine
- Extrathyroid syntheses

THE CIRCULATING THYROID HORMONES
- The iodine of the protein bound iodine
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THE CHEMISTRY OF THE THYRONINE-BINDING PROTEINS

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Consideration of hormones in the blood requires an integration of many aspects of hormonal economy In the passive sense blood constitutes a transport phase which is interposed between the synthesis of hormone at glandular sites and the degradation of hormone by peripheral structures As such the concentration and the nature of circulating hormone at any given time represent the composite of biosynthetic and catabolic interactions In a more active sense the blood provides multiple plasma proteins which can form associations of varying specificity with hormones in the blood Thus for many hormones the blood constitutes not merely a passive aqueous diluent but rather a medium in which binding interactions can occur which may confer anatomic specificity as well as limitations in rate upon the cellular delivery of hormone

For the hormones of the thyroid gland recent progress has been enormous in all of these areas Extensive review is beyond the scope of this chapter hence discussion is largely confined to data which have been obtained in man Since thyroxine constitutes the principal product of thyroid biosynthesis and the major circulating thyroid hormone it is proposed to describe the normal formation glandular release transport and turnover of thyroxine in detail in order to provide a framework upon which may be projected a briefer discussion of other normal and abnormal products of glandular biosynthesis and peripheral degradation

THE BIOSYNTHESIS OF THYROID HORMONES

Intrathyroid Metabolism of Iodine

The thyroid gland contains in unique juxtaposition a specific macromolecular glycoprotein thyroglobulin (Heidelberger and Pedersen 1935) and a mechanism for the efficient concentration of ionic iodide Little is known about the formation of thyroglobulin and the homogeneity of thyroglobulin only recently has been questioned (Ingbar *et al* 1959) However with the advent of I^{131} and powerful chromatographic techniques there has been extensive documentation of the fate of iodine within the thyroid gland Analysis of the latter phenomena may be simplified by dividing the intrathyroid metabolism of iodine into four sequential steps

The first step consists of a selective extraction of ionic iodide from the extracellular fluids and the establishment of concentration differentials for iodide between the thyroid gland and the plasma

(Schachner *et al* 1944 Franklin *et al* 1944 Vanderlaan and Vanderlaan 1947 Taurog *et al* 1947) Thereby a concentrated substrate is provided for hormonogenesis The term thyroid iodide trap which has been applied to this process bears the connotation of a unidirectional flux and is therefore misleading It has been shown that energy derived from intrathyroid oxidative metabolism is required for both the establishment and the maintenance of thyroid plasma iodide gradients (Slingerland 1955 Freinkel and Ingbar 1955a b) Concentration differentials are greatest when further formation of hormones is blocked by such agents as the thiourea derivatives (Stanley and Astwood 1949) However the gradients are not reduced to unity even in the unblocked thyroid gland (Ingbar and Freinkel 1956) Clearly iodide transport is the principal determinant of net hormone synthesis However the presence of manifest iodide gradients in the unblocked thyroid gland would suggest that the rates of reactions subsequent to concentration of iodide may also be of rate limiting significance

The oxidative formation of iodinated tyrosines and thyronine within the matrix of the thyroglobulin molecule is the second step in hormonosynthesis Extrapolation from chemical experience would indicate that a conversion of iodide to molecular iodine must precede organic iodinations Whether this oxidation is mediated by a specific enzyme such as peroxidase (Astwood 1954) or whether it is conditioned by the prevailing oxidation reduction potential within the thyroid gland (Roche and Michel 1955) has not been resolved In either circumstance the evolution of iodine would be accompanied by an immediate iodination of tyrosyl groups in the thyroglobulin molecule The initial product of iodination is L 3 monoiodotyrosine and recently the catalytic participation of a specific enzyme tyrosine iodinase (Fawcett and Kirkwood 1954) has been implicated Formation of monoiodotyrosine is a hardy function It is the first organic iodination to become manifest during the embryological development of the thyroid gland (Trunnell and Wade 1955) and it is retained by fortified thyroid homogenates and stored thyroid slices which have lost the capacity to concentrate iodide or to perform more complex iodinations (Mayer *et al* 1956) The kinetics of hormonal biosynthesis have been clarified by isotopic data It has been demonstrated that L 3 monoiodotyrosine is the precursor of L 3 5 diiodotyrosine and that L 3 5 3 5 tetraiodothyronine or thyroxine is derived from diiodotyrosine (Chaikoff and Taurog 1948 Roche and Michel 1953) In

formation concerning the precise biochemical pathways is meager and largely derived from analogies to other systems At present the early suggestion of Harrington (Harrington and Barger 1927 Harrington 1944) that thyroxine is formed by the oxidative condensation of two molecules of diiodotyrosine with the loss of one alanine side chain continues to be a tenable thesis Within this framework and in the absence of a convincing demonstration of intrathyroid dehalogenation of thyronine derivatives the same mechanism may be invoked to explain the biosynthesis of the more recently described thyroid hormones Thus L 3 5 3 triiodothyronine (Gross and Pitt Rivers 1952 1953 Roche *et al* 1952b) which has been found in the thyroid glands of all species and such compounds as L 3 3′ diiodothyronine and L 3 3′ 5 triiodothyronine (Roche *et al* 1955) which have been identified in enzymatic hydrolyzates of rat thyroid glands could arise during the conjugation of L 3 monoiodotyrosine and L 3 5 diiodotyrosine in varying combinations (Roche and Michel 1955 Michel 1956) Alternative biosynthetic possibilities have not been excluded As yet distinct enzyme systems for the formation of diiodotyrosine and for the oxidative condensation of iodinated tyrosines have not been isolated The presence of monoiodohistidine a compound which seemingly subserves no function in the biosynthesis of hormone but which may account for 2 per cent of the total organic iodine of the rat thyroid gland would suggest that intrathyroid iodinations need not be entirely specific (Roche *et al* 1952a)

Refined analyses of the composition of human thyroid tissue have recently been performed (Braasch *et al* 1955) The average percentile distribution of I^{127} in trypsin hydrolyzates of biopsies of normal thyroid glands was as follows thyroxine 35 per cent triiodothyronine 8 per cent diiodotyrosine 25 per cent and monoiodotyrosine 17 per cent *The mean distribution of iodine in untreated goitrous glands* was not significantly different

All of the above substitutions and molecular rearrangements seemingly occur within the matrix of the thyroglobulin molecule (Ludwig and von Mutzenbecker 1936 Pitt Rivers 1948) Although the anatomical site for the interactions has been varyingly localized to the apical portion of the epithelial cells and the intrafollicular lumina there is general agreement that the iodinated thyroglobulin is stored as colloid within the follicles (Leblond and Gross 1948 Doniach and Pelc 1949 Wollman and Wodinsky 1955) Approximately 5 to 10 mg of organic iodine are normally present within the human thyroid

gland (Gutman et al 1932 Riggs 1952 Berson and Yalow 1954 Ingbar et al 1954 Braasch et al 1955 Nodine et al 1957) On the other hand under conditions of normal dietary intake and thyroid function the net daily assimilation of iodide by the thyroid is equivalent to about 50 to 100 μg (i e about one third of the extrathyroid pool of ionic iodide) (Stanley 1949 Riggs 1952 Burrows and Ross 1953 Ingbar and Freinkel 1955 Berson 1956) The large size of thyroglobulin precludes its release into the circulation except in situations where the integrity of the follicular epithelium has been disrupted such as during surgery (Lerman 1940) following radiation (Tong et al 1952 Robbins et al 1952 1954 Robbins 1954) or during the course of subacute (Ingbar and Freinkel 1958a) or chronic thyroiditis (Gribetz et al 1954 Roitt et al 1956 Doniach and Roitt 1957 White 1957) Thus it is readily apparent that the stored thyroglobulin constitutes a large reservoir of hormone active amino acids which is interposed between the synthesis and the elaboration of thyroid hormone Proteolytic disruption of the thyroglobulin and liberation of hormone from peptide linkage constitutes the third phase of thyroid hormonosynthesis (De Robertis 1949) This is effected by an enzyme (De Robertis 1941) which has many of the characteristics of a cathepsin and which has been isolated in relative purity (McQuillan et al 1953)

Disposition of the free iodinated amino acids which are liberated by enzymic proteolysis (Alpers et al 1955) may be viewed as the final step (step four) in the intrathyroid metabolism of iodine Mono and diiodotyrosine are deiodinated by glandular dehalogenase and the resultant ionic iodide is available for intrathyroid recycling and incorporation into hormone (Roche et al 1952c 1953 Querido et al 1956) This may account at least in part for the failure of the substituted tyrosines to appear in the peripheral circulation The dehalogenating enzyme does not act upon the iodothyronines (Roche et al 1953) Thus the hormone active products of proteolysis can directly enter the circulation presumably by diffusion or by binding interactions with the thyroxine binding proteins in the perfusing plasma (Ingbar and Freinkel 1957) Direct characterization of the thyroid venous effluent has revealed that thyroxine constitutes the principal hormone secreted by the thyroid gland (Taurog et al 1956b)

All of the discrete phases of hormonogenesis are stimulated by pituitary thyrotropin (TSH) (Taurog et al 1958a) By pharmacological dissection of isolated functions it has been shown that TSH

can enhance the accumulation of iodide (Vanderlaan and Greer 1950 Halmi *et al* 1953 Wollman and Scow 1953) the iodination of tyrosine and the oxidative condensation to yield thyronines (Morton *et al* 1942 Chaikoff and Taurog 1949 Taurog *et al*, 1958b c) and the proteolytic release of thyroid hormones (Keating *et al*, 1945 Rawson 1949 Olin Lamberg and Lamberg 1953 Wahlberg 1955) The magnitude of response is conditioned by the functional state and iodine content of the thyroid gland and by the previous diet (Halmi and Spirtos 1954 Vanderlaan and Caplan 1954) The multiplicity of effects of TSH upon hormonogenesis would suggest that the changes in iodine economy are secondary to a more basic action upon some aspect of glandular intermediary metabolism (Freinkel 1957 Taurog *et al* 1958b) Although some beginning attempts have been made to elucidate the metabolic pathways of thyroid tissue (Freinkel 1958) this still constitutes a relatively unexplored area of investigation

Extrathyroid Syntheses

Certain of the steps of thyroid hormone biogenesis are also found in extrathyroid sites Thus ionic iodide is concentrated in the stomach salivary glands placenta and lactating mammary glands (Lipschitz 1929 Schiff *et al* 1947 Myant *et al* 1950 Honour *et al* 1952 Freinkel and Ingbar 1953 1956 Logothetopoulos and Scott 1955) Moreover in these areas as in the thyroid gland iodide transport can be inhibited by such monovalent anions as ClO_4^- and SCN^- (Freinkel and Ingbar 1953 1956 Rowlands *et al* 1953) Extrathyroid organic iodinations have also been described Iodinated proteins can be formed by *in vitro* preparations of salivary mammary and splenic tissue (Freinkel and Ingbar 1956 Taurog *et al* 1956a) and circulating protein bound iodine may be increased by the administration of massive quantities of iodine to athyreotic human beings (Danowski *et al* 1950a) However it does not seem likely that the availability of hormone active moieties is appreciably influenced by extrathyroid synthesis Although some labeled thyroxine may be recovered from the blood of thyroidectomized rats given inorganic I^{131} (Morton *et al* 1943) and thyroidectomy cells in the pituitary glands of these animals can be partly reversed by the administration of pharmacological amounts of iodide (Hum *et al* 1951) none of the phenomena results in a restoration of eumetabolism Similarly in human beings post thyroidectomy myxedema is sufficiently common (Asper and Wiswell

1956) to reinforce the impression that extrathyroid sites do not materially contribute to circulating thyroid hormone

THE CIRCULATING THYROID HORMONES

Although the ionic iodide of blood constitutes the critical substrate for the synthesis of thyroid hormone its absolute concentration is vanishingly small Ionic iodide has been indirectly estimated to account for less than one tenth of the total serum iodine under normal circumstances (Riggs 1952) By far the overwhelming preponderance of the iodine of serum is organic in nature Several chemical characteristics of this organic iodine have been known for some time Early reports indicated that it was nondialyzable did not pass an ultrafilter and could be precipitated quantitatively with the plasma proteins (Trevorrow 1939 Man *et al* 1942 Taurog and Chaikoff 1948) Moreover thyroid activity and the iodine content of washed plasma protein precipitates were closely correlated (Salter *et al* 1941 Salter 1949) It was also demonstrated that the precipitated iodide was not in peptide linkage since it could be extracted from unhydrolyzed plasma with butanol acetone or ethanol (Trevorrow 1939) Thus almost two decades ago it was realized that measurable quantities of thyroid hormone existed in the blood that circulating hormone differed from the intrathyroid thyroglobulin and that it was transported in some form of physicochemical union with plasma proteins As such the hormone of the thyroid gland constitutes the historical prototype for the concept of an association between plasma proteins and endocrine secretions This concept is now so firmly established that synonyms for circulating thyroid hormone such as serum precipitable iodine (SPI) or protein bound iodine (PBI) have become standard clinical parlance

*Both the iodine and the protein components of the associated com*plex have been characterized These are discussed separately in the section to follow

The Iodine of the Protein bound Iodine

In contradistinction to ionic iodide which is freely diffusible into the water of red blood cells (Wallace and Brodie 1937) the organic iodine of the blood is excluded from the circulating erythrocyte (Riggs *et al* 1942 Ingbar *et al* 1954) Characterization of this organic iodine by classic chemical techniques requires brief consideration of the

solubility properties of the iodinated moieties which are normally present within the thyroid When thyroxine is added to plasma 90 per cent of it is recoverable in a zinc hydroxide precipitate and more than 95 per cent is precipitated by trichloracetic acid (Tong *et al* 1954) Similar precipitation relationships obtain for triiodo thyronine (Blizzard and Mosier 1957) On the other hand the pH of the precipitation procedure governs the fate of tyrosines added to plasma (Blizzard and Mosier 1957 Acland, 1958) Thus whereas less than 10 per cent of added mono or diiodotyrosine is precipitated with the plasma proteins by trichloracetic acid more than 50 per cent can be recovered in a Somogyi precipitate presumably due to loose associations between the tyrosines and albumin at alkaline pH (Acland 1958) The iodinated tyrosines and thyronines can be extracted from protein precipitates or acid aqueous solutions by n butanol (Leland and Foster 1932 Blau 1933 Trevorrow 1939) Re extraction of the butanol by an alkaline reagent (Blau 1933) recovers the tyrosines but not the thyronines Thyroglobulin which can be quantitatively re covered by all techniques of protein precipitation is insoluble in butanol acid and thus is readily distinguished from the other com pounds

Most if not all of the organic iodine of blood is composed of compounds whose solubility characteristics resemble those of the hormone active thyronines Thus 70 to 90 per cent of the total serum iodine is extractable with butanol and not re extractable with alkaline Blau s reagent (Taurog and Chaikoff 1948 Man *et al* 1951) A larger percentage can be precipitated with the plasma proteins (Danowski *et al* 1950a b Man *et al* 1951) Available analytical methods are not sufficiently refined to assess whether the difference between extraction and precipitation techniques constitutes a true phenomenon or a fractionation artifact (Acland 1958)

More definitive identification of the chemical nature of the iodine in protein bound iodine has been possible by the conjoint usage of isotopes and chromatography (Roche *et al* 1954a) This has afforded a degree of resolution which was precluded in conventional chemical analysis of stable serum iodine In man as well as in other mammalian species thyroxine accounts for 80 to 90 per cent of the total organic I^{131} which appears in the serum following the administration of in organic I^{131} (Taurog and Chaikoff 1948 Laidlaw 1949 Rosenberg 1951 Taurog *et al* 1958a) Although labeled L 3 3 diiodothyronine

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and L 3 3 5 triiodothyronine have been observed in rat serum (Michel 1956) L 3 5 3 triiodothyronine is the only organic iodinated compound besides thyroxine that has been convincingly demonstrated in the circulation of normal man (Gross and Pitt Rivers 1952 Benua *et al* 1955) by combined isotopic and chromatographic methods

However it should be recognized that these techniques provide no true estimate of the percentile contribution of the individual thy ronines to total chemical serum iodine since thyroxine and tri iodothyronine may differ in their relative specific activities at the time of release from the thyroid gland and do differ in their extrathyroid rates of turnover (Sterling *et al* 1954) True quantitation would re quire not only chromatographic resolution and radioactive assay but also chemical estimation of the iodine content of the individual com ponents Existing microanalytical techniques preclude such refine ments although the limits of sensitivity are being constantly improved (Bowden *et al* 1955) Thus for the moment the absolute concentra tion of thyroxine and L 3 5 3 triiodothyronine in plasma cannot be defined with certainty Moreover the possible contribution of other iodinated compounds to the circulating organic iodine cannot be wholly excluded Conceivably such moieties may be discovered as chroma tographic techniques are improved and newer solvent systems achieve increasingly discriminatory chromatographic separations What is the *origin of the circulating thyroxine and L 3 5 3 triiodothyronine?* Thyroxine originates within the thyroid gland Presumably the thy roid gland is also the major source of the triiodothyronine since the early evidence for its peripheral origin has been recently refuted (Las siter and Stanley 1958)

In theory however a possible contribution of the products of periph eral degradation to the circulating organic iodine cannot be dis missed The quantitatively minor importance of such compounds need not parallel their metabolic significance Thus consideration of the metabolic fate of the thyroid hormones is appropriate

Urinary excretion of thyroid hormones accounts for negligible losses except in the presence of proteinuria (Recant and Riggs 1952 Rasmussen 1956 Robbins and Rall 1957) In man (Johnson and Beierwaltes 1953) in contradistinction to the rat (Taurog *et al* 1951 Albert and Keating 1952) only a small proportion of the extra thyroid hormone participates in an enterohepatic circulation Some of this is conjugated on the phenolic hydroxyl with glucuronic acid

(Taurog *et al* 1952 Roche *et al* 1954b) and subsequently hydrolyzed in the intestinal tract and reabsorbed The remainder approximately 20 per cent of the secreted thyroid hormone is excreted as hormonal iodine in the feces (Myant and Pochin 1950 Berson and Yalow 1954 Ingbar and Freinkel 1955) By far the greatest removal of extra thyroid thyroid hormones occurs as a consequence of structural degradation in the peripheral tissues Two main catabolic processes have been delineated (1) deiodination of the diphenyl ether nucleus and (2) oxidative deamination and decarboxylation of the alanine side chain to yield the pyruvic and acetic acid derivatives of the iodinated thyronines In support of the former enzymatic mechanisms for the partial deiodination of thyroxine to triiodothyronine have been demonstrated in slices of rat kidney (Albright *et al* 1954) and 3 3 diiodothyronine has been recovered in extracts of kidney tissue following the administration of labeled 3 5 3 triiodothyronine* to thyroidectomized rats (Roche *et al* 1956) Moreover active enzymatic deiodination of thyroxine has been effected with homogenates of a variety of rat tissues (Sprott and MacLagen 1955) The available evidence for the successive transformation of the alanine residue to iodothyropyruvic acid (by oxidative deamination) and then to iodothyroacetic acid (by oxidative decarboxylation) is more inferential although consonant with the metabolic fate of amino acids in general In thyroidectomized rats given labeled 3 5 3 triiodothyronine 3 5 3 triiodothyropyruvic acid has been recovered from bile and urine (Roche *et al* 1954c) and 3′ 3 5 triiodothyroacetic acid from extracts of kidney tissue (Roche *et al* 1956) In addition an enzyme capable of converting thyroxine and triiodothyronine to their respective acetic acid analogues *in vitro* has been isolated from rat kidney (Albright *et al* 1956)

It is of interest that all of the above degradation products display some hormonal activity As yet it is not known whether similar transformations occur in man and if so whether oxidative degradation of the alanine residue precedes or accompanies deiodination Only deiodination has been demonstrated with certainty because inorganic I^{131} is the only degradation product that appears in the serum of athyreotic human beings who have been given I^{131} labeled thyroxine or triiodothyronine This does not exclude the possibility of other conversions since degradation products might not spill over into the circulation because of tissue fixation at the sites of degradation

* For all iodinated amino acids L is understood unless otherwise stated

Alternatively they might be removed from the circulation with sufficient rapidity to escape detection by present techniques

When thyroxine is administered to thyroprival subjects considerable time precedes the onset of hormonal action The lag phase is appreciably shorter when L 3 3 5 triiodothyronine or certain of the other thyronine derivatives are given This has evoked the suggestion that thyroxine must be converted to an active form before it can exert hormonal action As yet there are no definitive data to support this thesis and no evidence has been presented to indicate that hormonal action and hormonal degradation are more than fortuitously related It is conceivable that the reported qualitative and temporal differences between the effects of thyroxine and other thyronine derivatives may reflect variations in their binding interactions with tissue or plasma proteins

The Protein of the Protein bound Iodine

The interaction between thyroxine and plasma proteins was not defined until the advent of zone electrophoresis Earlier efforts based upon dialysis salting out and moving boundary electrophoresis were largely unrewarding (Gley and Bourcet 1900 Trevorrow 1939 Taurog and Chaikoff 1948 Silver and Reiner 1950) However on the basis of iodine nitrogen estimations of human plasma fractions prepared by Method 6 of Cohn it was suggested that there were specific proteins for thyroxine binding concentrated in Fraction IV (the α globulins) and Fraction V (principally albumin) (Salter 1944 1949) These predictions were substantiated in 1952 when it was demonstrated that radioactive thyroxine migrated with the α globulins during electrophoresis of sera on filter paper in Veronal buffer at pH 8 6 (Gordon *et al* 1952) In the same year several groups of investigators localized the site of thyroxine protein association even more specifically to an area intermediate between the α_1 and α_2 globulins (Larson *et al* 1952 Robbins and Rall 1952 Winzler and Notrica 1952 Deiss *et al* 1952 Horst and Rosler 1953) The inter alpha protein with which thyroxine is associated during zone electrophoresis in barbital buffer at pH 8 6 has since come to be known as the thyroxine binding protein (TBP) or thyroxine binding globulin (TBG) of plasma In the Cohn fractions of plasma TBG is maximally present in Fractions IV-6 and IV 9 (Freinkel *et al* 1955) By zone electrophoresis TBG has been also demonstrated in normal and abnormal extravascular fluids (Alpers and Rall 1955 Freinkel *et al* 1957 Robbins and Rall 1959) thus

indicating that like other plasma proteins it is distributed in a portion of body water which includes freely exchanging intra and extra vascular components

In the absence of pure TBG an extensive body of information was accumulated by labeling the thyroxine binding components of serum either *in vivo* or *in vitro* with I^{131} labeled thyroxine and following the behavior of the radioactivity during filter paper electrophoresis in barbital buffer at pH 8 6 The validity of the indirect approach was substantiated by the demonstration that endogenously synthesized and exogenously added thyroxine were in ready exchange equilibrium (Albright *et al* 1955 Freinkel *et al* 1955) It was soon learned that although the major portion of tracer concentrations of labeled thyroxine was associated with TBG progressively larger fractions of labeled thyroxine became associated with albumin as the concentration of stable thyroxine in the serum was increased Moreover the displaced thyroxine could be recovered onto TBG by decreasing the concentration of thyroxine or by increasing the availability of TBG (Freinkel *et al* 1955) Thus TBG and albumin were assumed to be the primary and secondary carriers of thyroxine respectively and the distribution of thyroxine among plasma proteins was formulated in terms of a simple two-component binding interaction between TBG and albumin Recent developments have justified the early reservations about this simple formulation Within the past year a third thyroxine binding component has been identified (Rich and Bearn 1958 Ingbar 1958) and isolated from plasma fractions rich in TBG (Ingbar 1958) This protein could be demonstrated during electrophoresis of fractions in barbital buffer at pH 8 6 wherein it displayed a characteristic anodal migration approximately 20 per cent faster than that of serum albumin (Ingbar 1958) Accordingly it has been designated as the thyroxine binding pre albumin (TBPA) Such binding of thyroxine by proteins migrating ahead of albumin in barbital buffer had already been noted in cerebrospinal fluid (Alpers and Rall 1955) and in the serum of normal and nephrotic patients but its significance was not understood (Robbins *et al* 1957) Indeed when thyroxine migrated in the pre albumin zone during starch gel electrophoresis of normal serum this was interpreted as evidence for an alteration of the migration of TBG by the electrophoretic procedure (Rich and Bearn 1958) However it is now apparent that TBPA constitutes a distinct and physiologically meaningful normal serum component In concentrated solutions its thyroxine binding can be

demonstrated with ease during conventional zone electrophoresis in barbital-buffered systems. At normal serum concentrations it cannot be regularly demonstrated in this fashion, possibly because of protein interactions or trailing on the supporting medium. These methodological difficulties can be circumvented by using, instead of Veronal or phosphate buffer, a buffer system containing tris-hydroxymethyl amino methane and maleic acid (tris-maleate) (Ingbar, 1958). During electrophoresis of serum at pH 8.6 in the tris-maleate system, radioactive thyroxine is apportioned between TBG, albumin and TBPA. The contribution of TBPA to total thyroxine binding is appreciable. At physiological concentrations of thyroxine, the percentage of serum thyroxine which is associated with prealbumin is as great, or nearly as great, as that which is associated with the inter-alpha thyroxine-binding globulin. Thus, on the basis of the most recent information, at least three separate plasma proteins, TBG, TBPA and albumin, can be implicated in the normal transport of thyroid hormone in man, and any model of plasma thyroxine-binding interactions must be projected into more complex dimensions than had hitherto been suspected.

In lower mammalian species the plasma transport of thyroxine may differ. During electrophoresis of monkey, hog, horse, calf, lamb, sheep and steer sera in barbital buffer at pH 8.6 in the authors' laboratory, thyroxine migrates with the α-globulins and is progressively displaced onto albumin as the absolute concentration of thyroxine is increased. Under similar conditions endogenous thyroxine of dog serum is partitioned between the α- and β-globulins as well as albumin. It has not been possible to demonstrate multiple thyroxine-binding components in mouse, rat, guinea pig and rabbit sera. In the sera of these species thyroxine invariably migrates with the slowest moving portion of the albumin during electrophoresis in Veronal or phosphate buffer, and displacement onto secondary carriers is not effected by enriching the plasma with thyroxine. Although similar findings have been reported by others (Robbins and Rall, 1957), some authors using identical techniques have described the presence of a specific thyroxine-binding α-globulin in rat serum (Van Arsdel and Williams, 1956; Myant, 1957). The manifest differences in the species in thyroxine-plasma protein associations would suggest that caution must be exercised in extrapolating animal data to human beings, especially in those areas wherein thyroxine-binding interactions may be of significance.

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THE CHEMISTRY OF THE THYROXINE-BINDING PROTEINS

The recent suggestion that there are two proteins in plasma with especially great avidity for thyroxine (TBG and TBPA) necessitates that pre existing data concerning the properties of the thyroxine binding protein be reassessed (Ingbar 1958) *

It is necessary however to examine more carefully the evidence which indicates that TBG and TBPA are in fact distinct entities and that both are present in whole native plasma It has recently been reported that during starch gel electrophoresis of serum containing labeled thyroxine all radioactivity was confined to an area anodal to albumin or to albumin itself (Rich and Bearn 1958) No radioactivity was noted in the inter alpha position commonly associated with TBG It was concluded that during starch gel electrophoresis TBG migrated to the pre albumin zone This interpretation was consistent with initial observations in this system which indicated that globulins might in part migrate in this zone The possibility that the rapidly migrating thyroxine might be bound to at least two proteins with coincident migration was not considered Shortly thereafter it was reported that during paper electrophoresis in tris maleate buffer at pH 8 6 radio active thyroxine is associated with a pre albumin (TBPA) with TBG and with albumin (Ingbar 1958)

These apparently discrepant observations require resolution Several questions are encompassed by this problem First does TBPA exist as a protein distinct from TBG in native plasma i e might TBPA be an artifact of electrophoresis or might it represent a dissociated frag ment of TBG? Alternatively might TBG represent TBPA which has undergone an interaction with another plasma protein and thereby been altered in electrophoretic mobility? Second if TBPA does in fact exist in plasma why has its presence gone relatively unnoticed when thyroxine binding was studied by techniques which readily demon strated TBG?

Proteins migrating anodal to albumin have been noted in serum and cerebrospinal fluid during free electrophoresis in a variety of buffers (Fisk *et al* 1951 Hoch and Chanutin 1953) A review of the

* In one patient with carcinoma of the thyroid gland binding of both endo genously synthesized and exogenously introduced radioactive thyroxine to a protein similar to γ globulins has been described (Robbins *et al* 1956) Although of considerable interest this finding does not bear prominently upon the question of hormonal binding in the serum of normal subjects or of patients with most disease states

literature in this regard has recently been presented (Schultze *et al* 1956-57) The identity of any of these moieties with TBPA has not been completely verified However endogenously synthesized and exogenous I^{131} labeled thyroxine *have been shown to bind to the pre albumin* of cerebrospinal fluid during paper electrophoresis in Veronal buffer (Alpers and Rall 1955 Robbins and Rall 1957) Thus considerable binding avidity would appear to be present in the principal pre albumin of spinal fluid Workers in Germany have reported the virtual isolation of a pre albumin from pooled human plasma (Schultze 1956-57) Many of the chemical and physicochemical properties of this protein are similar to those of TBPA Antisera prepared by these workers against the largely purified protein reacted during immuno electrophoresis in phosphate buffer agar gel with a pre albumin com ponent of normal unfractionated serum Such antisera also react with the pre albumin of spinal fluid

The concentration of TBPA in plasma appears to be sufficiently small so that as is the case with TBG no discernible band appears in the pre albumin zone when routine paper electrophoretograms are stained with bromo phenol blue or Amido Schwarz However when large quantities of serum are subjected to paper electrophoresis in Veronal buffer protein staining does reveal a distinct pre albumin band The electrophoretic migration of this component is similar to that of TBPA and in a number of disease states its apparent con centration (as measured by Amido Schwarz) undergoes quantitative alterations similar to those which occur in the thyroxine binding capacity of TBPA (Aly and Niederhellmann 1958)

Finally when serum undergoes electrophoresis even in barbital buffer following addition of certain analogues of thyroxine which as is noted below (p 530) bind to TBPA even more strongly than does thyroxine migration of the radioactive compound in the pre albumin zone can regularly be demonstrated (Ingbar 1959)

These varied observations indicate that unless electrophoresis itself or the preparative procedures employed in the isolation of the pre albumins induces denaturing effects on the plasma proteins then TBPA must be present in whole native plasma

If TBPA is indeed a native protein of plasma it is remarkable that its function as a thyroxine binding protein was not recognized during earlier studies of TBG However binding of thyroxine by moieties migrating ahead of albumin even in Veronal buffer has been re peatedly observed particularly in certain pathological sera and in

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the binding of thyroxine by TBPA little if at all Veronal inhibits binding markedly though not completely Lack of complete inhibition of binding by Veronal is evident from the observation that purified preparations of TBPA in which the concentration of protein is higher than 0 5 gm per hundred milliliters continue to display avid binding of thyroxine even when electrophoresis is performed in Veronal buffer (Ingbar 1959)

Considerable evidence has been brought to bear upon the question of whether TBG and TBPA are distinct entities (Ingbar 1959) As noted earlier TBPA might represent a portion or prosthetic group of TBG dissociated by electrophoresis or other preparative procedures Alternatively TBG might consist of TBPA bound to another protein possibly an α_2 globulin As assayed by electrophoresis in tris maleate the binding capacities of TBG and TBPA may vary independently in a variety of clinical states For example the binding capacity of TBG which is markedly increased during pregnancy changes but little during the first few days post partum while the binding capacity of TBPA which is essentially normal prior to delivery decreases markedly during this time interval Similarly during therapy of thyrotoxicosis the binding capacity of TBPA increases from low to normal levels while the binding capacity of TBG remains essentially unchanged In addition recent studies of one patient revealed no demonstrable binding of thyroxine by TBG although the binding capacity of TBPA was normal Further evidence is provided by studies of the differential binding of various labeled congeners of thyroxine Thus during electrophoresis in tris maleate D thyroxine and L thyroxine bind to TBG equally well but the former does not bind to TBPA The same qualitative differences obtain with L triiodothyronine On the other hand the acetic and propionic acid analogues of thyroxine and triiodothyronine bind to TBPA with varying affinities but evidence no binding to TBG Finally as noted earlier Veronal appears to inhibit the binding of thyroxine by TBPA but has little effect on binding by TBG

Further data in these areas continue to accumulate rapidly Although it is apparent that complete proof that TBPA is a distinct and native component of plasma remains to be obtained the weight of present evidence suggests that it is Recent developments however indicate that considerable caution need be employed in interpreting electrophoretic data New techniques may reveal new and unexpected complexities in the binding of thyroid hormones in plasma

cerebrospinal fluid (Robbins and Rall 1957) The finding though noted was paid no further heed

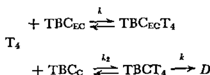
It remains nevertheless to consider why TBPA is not regularly demonstrable in normal serum during electrophoresis in Veronal buffer Two possibilities seem particularly likely The rapid anodal migration of TBPA at pH 8 6 would make it particularly susceptible to trailing on paper This together with the presumed low concen tration of the protein in plasma would combine to prevent the majority of the protein from achieving its characteristic migration Demonstration of stainable pre albumin following electrophoresis in Veronal buffer when large quantities of serum are applied to one spot is consonant with this explanation Tris maleate buffer by re ducing trailing to paper might permit migration of a greater fraction of TBPA to its characteristic position Recent experiments suggest that some trailing of pre albumin occurs even in the tris maleate system When nephrotic serum proteins were reconstituted at a con centration of 2 0 gm per hundred milliliters and enriched with radioactive thyroxine no TBPA was demonstrable following electro phoresis of 20 microliters however TBPA could clearly be seen when 50 microliters were used In whole normal serum trailing of TBPA in the tris maleate system appears to be slight since the binding capacities of individual sera did not differ appreciably when electrophoresis of 20 microliter and 50 microliter samples was performed (Ingbar 1959)

However it seems unlikely that adsorption to paper alone can account for failure to find TBPA more consistently in serum under going electrophoresis in Veronal buffer Tetraiodothyroacetic acid is bound to TBPA even more strongly than is thyroxine When the I^{131} labeled analogue is added to serum and electrophoresis in Veronal buffer is then performed binding by TBPA is readily seen Trailing seems to be slight However the binding capacity of normal serum for this analogue is greater when determined in tris maleate than in Veronal buffer In addition when solutions of purified TBPA dis solved in tris maleate and containing radioactive thyroxine are dia lyzed against either human serum albumin or whole serum equilibrated with the same buffer thyroxine binding by TBPA is approximately twelve times greater than when comparable studies are performed in Veronal buffer Binding activity of TBPA in tris maleate is similar to that observed when dialysis experiments are performed in simple inorganic buffers These data suggest that while tris maleate inhibits

normal and abnormal extravascular portions of the extracellular fluids would indicate that the virtual volume of the body waters in which thyroxine is distributed (i e the thyroxine space) may be delimited by the thyroxine binding proteins Finally in early studies of thyroxine metabolism it was noted that the rate of disappearance of thyroxine from the blood stream was much slower than that of other amino acids and more in accord with values previously reported for certain of the proteins of plasma (Ingbar and Freinkel 1955) The speculation was then posed One may wonder whether the distribution and fate of thyroxine are related to the fate of the proteins to which it is bound (Ingbar and Freinkel 1955) As other thyronine derivatives have been examined it has become apparent that a rough *correlation does indeed exist between their biological half lives and* the intensities of their interactions with the thyroxine binding proteins

Attempts to assign more precise dimensions to the physiological importance of protein binding interactions in the metabolism of thyroid hormones must await resolution of the steps involved in the transcellular transfer of hormones To date this sequence has not been elucidated and the experimental efforts justify only inferential conclusions However since the general considerations may be equally extrapolated to other hormones which travel in association with plasma proteins some summation of the available information may be appropriate

A formulation has been recently proposed (Freinkel *et al* 1957) and this will be extended in order to interpret the data relevant to the role of binding phenomena in the turnover of thyroxine *in vivo* Herein



T_4 = thyroxine which is not associated with any binding components TBC_{EC}* = the sum of the circulating extracellular thyroxine binding components (i e TBG TBPA albumin and possibly others) TBC_C = the sum of the cellular thyroxine binding components (pre

*Methods for the direct estimation of TBC and TBPA are not yet available Thus in the present text TBC_C refers to values determined indirectly on the basis of the thyroxine binding capacities of the TBG and TBA in a measured aliquot of plasma or serum

Although both TBG and TBPA have been prepared in apparently homogeneous states only fragmentary data concerning their physical and chemical properties are presently available These are detailed in

TABLE I Several Physical and Chemical Properties of Thyroxine binding Globulin (TBG) and Thyroxine binding Pre albumin (TBPA)

Prop rty	TBG	TBPA (Ingb 19 9)	P alb min (Sch lt t l 19 6 57)
$S_{20\ w}$	3 3 (Robbi s and Rall 1957)	4 4	4 2
	4 1 (Ingbar et al 1957)	–	–
Isoelect i point	4 5 (Robbi s and Rall 1957)	≈ 4 5	–
Molecula w ight	–	–	61 000
Glycoprotei	Yes† (Ingbar t al. 1957)	No†	No
N rami c acid	Yes‡ (Ingba 1959)	No‡	No
Phosphoru	–	–	0 01%
S lf	–	–	0
N/dry w ight	10 2% (Ingba et al 1957)	–	–

Det mi ed whol rum
† Po ti PAS tai f pap l t ph t g m
‡ Alt ti i lect i po t f ll ng i ub ti w th b t i l m nid

Table I *Since a pre albumin similar to* TBPA *has been isolated* by independent techniques pertinent properties of this protein are also included

THE PHYSIOLOGICAL SIGNIFICANCE OF PROTEIN-BINDING INTERACTIONS IN THE TRANSPORT OF THYROID HORMONE

It has long been recognized that micromolecules can achieve certain of the attributes of macromolecules as a consequence of being bound to plasma proteins (*Bennhold 1932* Davis *1946*) *These macro* molecular characteristics are especially manifested in the (1) excretion (2) distribution and (3) transcellular flux of the micromolecule With regard to excretion negligible quantities of thyroxine are present in normal urine (Myant and Pochin 1950 Rall 1950) Thus it may be *inferred that* by *virtue of being tightly* bound to plasma proteins thyroxine in contradistinction to other amino acids does not freely cross the glomerular membrane Similarly the *distribution of thyroxine* resembles that of plasma proteins rather than that of other amino acids The recent demonstrations of the inter alpha thyroxine binding globulin (TBG) and the thyroxine binding pre albumin (TBPA) in

The Interaction of TBC_{EC} and T_4

With plasma or serum (Albright *et al* 1955 Freinkel *et al* 1955) and with fractions of plasma proteins (Freinkel *et al* 1955) it has been demonstrated that the distribution of thyroxine among the various components of TBC_{EC} is reversibly conditioned by the relative abundance of the individual binding components (such as TBG TBPA and albumin) and the amount of T_4 which is to be bound Although some thyroxine is associated with albumin at all levels of plasma enrichment the albumin bound fraction increases steeply as the binding capacities of TBG and TBPA are exceeded Saturation of albumin has not been observed at the thyroxine concentrations which have been examined Existing techniques have not revealed the presence of measurable quantities of unbound or free thyroxine T_4 However by analogy to other binding equilibria it seems a physico chemical *certainty that some thyroxine must exist in the unbound* state

Mathematical techniques have been devised for the indirect estimation of the free thyroxine and by this approach an average concentration of $4\ 7 \times 10^{-}$ μg per milliliter or 6×10^{-11} M has been derived for human serum (Robbins and Rall 1957) The proposed values must be viewed with some reservations First the calculations were based on the assumption that serum contains only two thyroxine binding components (TBG and albumin) The recent demonstration of thyroxine binding pre albumin TBPA would indicate that this is not the case Second all of the measurements were performed at pH 8 6 Similar binding maxima need not obtain at the physiological pH of 7 4 and indeed experiments to be cited below would suggest that the associations between TBC_{EC} and T_4 are reduced as the pH is lowered Third the mathematical treatment has been based on the assumption that TBG contains only a single binding site for T_4 and that the successively bound molecules do not interact Although this is a reasonable thesis it has not been proven Finally intrinsic to the calculation of unbound thyroxine in human serum are constants for the association between thyroxine and albumin which have been obtained with preparations of bovine albumin Such extrapolation from other species is hazardous and all the more so since the presence of a TBG contaminant has been demonstrated even in crystalline preparations of bovine albumin (Freinkel *et al* 1955)

An alternative method for an indirect estimation of free thyroxine

sumably protein in nature and as yet neither identified nor anatomically localized) $TBC_{EC}T_4$ and TBC_CT_4 = the absolute quantity of thyroxine *which is associated with* extracellular or cellular thyroxine binding components respectively and D = the absolute quantity of products derived from the degradation of thyroxine per unit time

The rate constants have been designated as follows k_1 and k_2 = the association constants between T_4 and TBC_{EC} or TBC_C respectively and k_3 = the fractional rate of degradation of cell bound thyroxine

Thus thyroxine turnover *in vivo* has been *formulated in terms of* two sequential steps (1) the cellular accumulation of hormone and (2) the cellular disposition of hormone * Within this format it is postulated that circulating thyroxine has the dual potentiality of *reversibly affixing to either cellular* or extracellular thyroxine binding components so that the static cellular/extracellular distribution of thyroxine conforms to a reversible binding equilibrium between these interactants Thereafter irreversible loss of thyroxine from the thyroxine space is effected as a constant proportion of cell bound thyroxine (TBC_CT_4) is irreversibly removed from the equilibrium by degradation at a given rate (k_3)

*Model systems have been employed to examine the individual por*tions of the hypothetical sequence The integrated sequence affords a convenient frame of reference for the review of these data and for attempts to extrapolate them to dynamic events *in vivo* Although the formulation has been presented in terms of the absolute quantities of the individual components it is obvious that binding interactions in any single area will occur on the basis of concentration per unit mass Where available data are best expressed on the basis of such concentration parentheses will be employed in the subsequent portions of the text – for example (TBC_{EC}) – to denote the concentration of extracellular thyroxine binding components per aliquot of extra*cellular fluid*

For purposes of simplicity the loss of unaltered thyroxine from the body through excretory process s has not been included in the model system Although fecal loss of thyroxine may be appreciable (Myant and Pochin 1950 Berson and Yalow 1954 Ingbar and Freinkel 1955) *there is at present no convincing evidence* that the fecal excretion of thyroxine in man is conditioned by binding phenomena Indeed the proportion of the daily thyroxine turnover which may be accounted for in the stool seem to vary only slightly in pathological states Binding interactions may affect the urinary excretion of intact thyroxine However except *in proteinuric* states (Recant and Riggs 1952 Rasmussen 1956 Robbins and Rall 1957) the quantitative importance of this urinary loss is vanishingly small

The Interaction of TBC_C TBC_{FC} and T_4 *in Vitro*

In the authors laboratory slices of beef and sheep liver kidney and heart (Freinkel *et al* 1957) and human placenta have been suspended for short periods in fixed volumes of media containing (1) various quantities of thyroxine and (2) protein fractions of varying TBPA TBG or albumin content During this time interval thyroxine is not significantly degraded in these systems Thus they afford models of the factors which delimit the static partition of thyroxine between cellular and extracellular phases Herein cellular uptake of thyroxine varies (1) inversely with the thyroxine binding capacity of the extracellular media (2) directly with the absolute amount of thyroxine within the systems and (3) directly with the cellular mass As might be anticipated from equilibrium principles the distribution of a constant amount of thyroxine can be predictably altered by varying the concentration of either the cellular binding or the extracellular binding reactants or the absolute amount of thyroxine which is to be bound If the thyroxine space within such systems is calculated on the basis of (total unaltered thyroxine) (thyroxine per milliliter of suspending medium) then the virtual volume of thyroxine distribution is smallest when the TBC_{EC} are most abundant The partition is conditioned by pH Cellular uptake of thyroxine from protein containing suspending media increases as the extracellular pH is reduced from pH 8 to pH 5 (Freinkel *et al* 1957) The *in vitro* systems also display stereochemical specificity Cellular uptakes of such agents as L triiodothyronine or D thyroxine which are less avidly bound by the thyroxine binding proteins of plasma (Larson and Albright 1955 Freinkel and Ingbar 1955) are greater than those of L thyroxine

Results similar to the above have been obtained in other *in vitro* systems in which human plasma has been substituted for more dilute solutions of purified plasma fractions as the extracellular thyroxine binding phase and isolated rat hemidiaphragms (Hogness *et al* 1957 Hamolsky *et al* 1957) or human red blood cells (Hamolsky 1955 Crispell *et al* 1956 Crispell and Coleman 1956 Hamolsky *et al* 1957) have been employed as the cellular thyroxine binding phase The erythrocytic system has received particular attention

Insofar as uptake of thyroxine by red blood cells from normal donors is increased in the presence of plasma from thyrotoxic patients and decreased in the plasma from myxedematous patients the red

in vivo has also been proposed (Robbins and Rall 1957) It is based on the observation that the small quantity of thyroxine which is normally present in urine cannot be quantitatively precipitated with the urinary proteins Thus if one assumes that the nonprecipitable thyroxine of urine originated during the trans glomerular passage of unbound thyroxine then the concentration of free thyroxine in plasma may be calculated by simple substitution in the clearance formula Once again however the assumptions underlying the mathematics are unproven It is equally conceivable that most if not all of the urinary thyroxine originally crossed the glomerulus in protein linkage at pH 7 4 but that (1) binding associations are less intense at the acidic pH and the enormous dilutions that obtain in voided urine or that (2) TBC_{EC} T_4 bonds were disrupted within the kidney during tubular acidification and the protein components were re absorbed disproportionately thereafter Clearly for the moment exact values cannot be given for the concentration of the hypothetical free thyroxine nor can it be said that the entity exists as more than a transitory phase during the transfer of thyroxine from one thyroxine binding component to another The current availability of pure human TBG TBPA (Ingbar 1959) and albumin holds promise that precise association constants may be derived and that more definitive answers will not be long in forthcoming

The Interaction of TBC_C and T_4 *in Vitro*

During incubation of tissue slices (Freinkel *et al* 1957 Hogness *et al* 1957) or red blood cells (Crispell *et al* 1956 Crispell and Coleman 1956) in saline media containing labeled thyroxine there is an accumulation of thyroxine from the extracellular phase which slowly approaches asymptotic values Considerable adsorption of thyroxine to glassware occurs *pari passu* (Freinkel *et al* 1955) Cellular uptake is retarded at reduced incubation temperatures but it is independent of cellular metabolic activity and it is uninterrupted by anaerobiosis or by prior boiling of the suspended tissue (Crispell and Coleman 1956 Freinkel *et al* 1957) As the concentration of extra cellular thyroxine is increased the percentile cellular uptake is also augmented (Crispell *et al* 1956 Freinkel *et al* 1957 Hogness *et al* 1957) Under all conditions most of the cell bound thyroxine can be reversibly recovered by the introduction of thyroxine binding plasma proteins into the suspending media (Crispell and Coleman 1956 Freinkel *et al* 1957)

mental procedure may *per se* modify TBC_C Nonetheless several striking similarities to the slice studies have been obtained

Initial efforts were directed toward changing (TBC_{EC} T_4) acutely Patients with treated myxedema were given tracer doses of I^{131} labeled thyroxine One week following equilibration of the labeled hormone throughout the thyroxine space a concentrated solution of TBPA and TBG (i e 12 gm of Cohn Fraction IV 6) was infused during a 30 minute interval (Freinkel and Ingbar 1955c) The acute elevation of (TBC_{EC}) was followed by an abrupt rise of the PBI^{131} of the serum for 12 to 24 hours possibly corresponding to a contraction of the thyroxine space In many ways the results simulated the findings in the reversibility experiments previously described in which thyroxine bound to tissue slices could be recovered into the suspending medium by the introduction of TBG and TBPA into the system The infusion experiments produced only a transient rise in (TBC_{EC}) To assess the effects of prolonged augmentation of (TBC_{EC}) upon thyroxine distribution patients with treated myxedema were given large quantities of oral estrogen for 4 to 6 weeks until the plasma levels of TBG were doubled Contrary to expectations the thyroxine space was not changed in these patients with estrogen induced elevations of (TBC_{EC}) in some instances it was actually increased (Dowling *et al* 1958) However since fluid retention often accompanied the estrogen regimen the possibility cannot be excluded that the apparently unaltered thyroxine space was relatively contracted when related to the expanded extracellular fluid volumes Moreover the effects of chronic estrogen administration upon TBC_C are unknown

Attempts have also been made to alter distribution phenomena by changing the concentration of T_4 Four milligrams of unlabeled thyroxine have been injected into human subjects 1 week following the equilibration of a tracer dose of I^{131} labeled thyroxine throughout the thyroxine space (Richards *et al* 1958) The acute tripling of plasma hormone levels resulted in a precipitous decline in serum PBI^{131} within ½ hour presumably reflecting an increase in thyroxine space and simulating the enhancement of cellular uptake which is effected *in vitro* when large quantities of thyroxine are added to the suspending medium (Freinkel *et al* 1957) In similar experiments performed by the authors and others (Sterling and Chodos 1956) in which only 2 mg doses of thyroxine were administered the thyroxine space was not altered Electrophoretic studies would

blood cell test has been advocated as a diagnostic test of thyroid status (Hamolsky 1955 Hamolsky *et al* 1957a b) From the foregoing it would seem likely that the red blood cell uptake test does afford a semi quantitative index of the concentration of TBG and TBPA in plasma In this *in vitro* system the cellular phase and extracellular fluid volumes are kept constant and the partition of tracer quantities of added I^{131} labeled thyroxine or triiodothyronine is conditioned by the endogenous concentration of protein bound iodine (PBI) and by the concentration of strongly binding (TBG and TBPA) and weakly binding (albumin) moieties in the plasma (Richards *et al* 1959) However diagnostic specificity of the red blood cell test is to be questioned Hypothyroid patterns are obtained in other conditions (Hamolsky 1955 Richards *et al* 1959) in which as in myxedema (TBC_{EC}) is elevated such as pregnancy (Dowling *et al* 1956 Robbins and Nelson 1958) or following estrogen therapy (Dowling *et al* 1956) and hyperthyroid patterns are obtained in other conditions (Hamolsky 1955 Richards *et al* 1959) in which as in thyrotoxicosis (TBC_{FC}) is reduced such as the reduced (TBG) in patients with nephrosis (Robbins and Rall 1957) and the depressed (TBPA) in patients with a variety of medical and surgical illnesses (Richards *et al* 1959)

The Interaction of TBC_C TBC_{EC} and T_4 *in Vivo*

By simple extrapolation from the above *in vitro* systems it may be postulated that during conditions of constant hydration and extra cellular fluid volume the measurement of the thyroxine space *in vivo* affords some index of the static partition of thyroxine between cellular and extracellular thyroxine binding components Thus an expanded thyroxine space might be anticipated if the binding equilibrium favored cellular thyroxine binding components whereas a contraction of the thyroxine space might ensue if the extracellular quantity of the thyroxine binding components were augmented

Experiments have been conducted in the authors laboratory designed to test the thesis that *in vivo* as *in vitro* thyroxine distribution may be formulated in terms of binding interactions between cellular and extracellular components For the moment the results must be interpreted with caution since (1) TBC_C cannot be sampled in the intact animal (2) the existence of TBC_C T_4 has not been demonstrated *in vivo* and (3) the possibility cannot be excluded that the experi

turnover may be accelerated in conditions in which (TBC_{EC}) and (TBC_{EC} T_4) are equally reduced so that inferentially derived values for T_4 are normal Such a relationship has been observed following the administrations of salicylates (Austen *et al* 1958)

In an effort to assign primary importance to one of the components of the binding sequence it has been proposed that (T_4) is the principal determinant of the net degradation of thyroid hormone i e the absolute formation of D (Robbins and Rall 1957) Recalculation of the experimental observations upon which this premise was based (Berson and Yalow 1954) would suggest that k_3 is regulated by (T_4) The many divergent situations outlined above in which k_3 varied independently of consistent directional changes in (TBC_{EC}) (TBC_{EC} T_4) or (T_4) afford strong evidence that this is not the case and that k_3 is largely if not entirely conditioned by factors intrinsic to the cell which are as yet unknown

The Formation of D

During homeostatis *in vivo* PBI remains constant This would imply that the irreversible loss of thyroxine from the thyroxine space through degradation and excretion is counterbalanced by the delivery to the thyroxine space of an equal amount of thyroxine from the thyroid gland Since the total phenomena are concerned with absolute amounts of thyroid hormone and since D represents the absolute quantities of degradation products that are formed per unit time it is obvious that any dynamic consideration of the role of binding interactions in hormone economy must be expressed in terms of D

Several rate limiting determinants of D warrant discussion As outlined earlier (p 538) the magnitude of cellular uptake of thyroxine constituted the first such factor In man the extrathyroid thyroxine space *comprises 15 per cent of body weight or something slightly less than* extracellular fluid *Although* focal areas of thyroxine concentration cannot be excluded the 15 per cent figure suggests that the static cellular/extracellular partition of thyroxine always favors the extracellular phase The rate constants for binding of thyroxine by extracellular components (k_1) and by cellular components (k_2) are sufficiently rapid *in vitro* (Freinkel *et al* 1955 1957 Robbins and Rall 1957) that it would seem unlikely that they could materially effect equilibration *in vivo* Thus on the basis of the values for thyroxine space one may surmise that (1) the quantity of cell

indicate that the latter studies failed because PBI was insufficiently elevated by the 2 mg doses to alter the *distribution of thyroxine* between the weakly binding (albumin) and strongly binding (TBG and TBPA) components of the TBC_{EC}

Alterations of k_3

In the above formulation of thyroxine turnover a constant proportion of cell bound thyroxine is irreversibly degraded per unit time The rate constant for this fractional removal of thyroxine from the cellular thyroxine space has been designated as k_3

There are many lines of evidence which suggest that k_3 may vary *independent of changes in extracellular thyroxine binding phenomena* During incubation of rat kidney slices in simple saline media labeled thyroxine is much more rapidly deiodinated by tissues obtained from thyrotoxic than from myxedematous donors (Larson *et al* 1955) Direct assessment of k_3 cannot be made *in vivo* since there is as yet no method for sampling TBC_C T_4 However some directional index may be obtained by observing the fractional rate of disappearance of labeled thyroxine from the plasma following distribution equilibrium throughout the thyroxine space Under conditions of normal binding interactions abnormalities in plasma disappearance rates should reflect aberrations of k_3 (*vide infra*) Thus during hypermetabolism induced by the chronic administration of triiodothyronine to euthyroid human beings the fractional rate of disappearance of labeled thyroxine from the circulation is increased even though (TBC_{EC}) is unaltered and both the measured values of (TBC_{EC} T_4) and calculated values of (T_4) are depressed (Richards *et al* 1958 Ingbar and Freinkel 1958b) * Other situations have been encountered by the authors in which fractional turnover of T_4 is accelerated despite normal values for (TBC_{EC}) (TBC_{EC} T_4) and by inference (T_4) These include (1) cold exposure in man (2) certain types of adolescent goiter (3) certain eumetabolic patients with treated Graves disease and (4) a significant proportion of *eumetabolic relatives of patients with* Graves disease (Ingbar *et al* 1956) In addition fractional peripheral

* Since distribution studies with TBG or TBA have not yet been performed the estimates of TBG and TBPA in aliquots of plasma or serum afford no index of the total quantity of extracellular thyroxine binding components TBC_C However on the basis of experience with other plasma proteins *in vivo* it does not seem unreasonable to assume that normal values for (TBC_C) indicate normal TBC_{EC} under conditions in which extracellular fluid volume and hydration are normal

are reduced Similarly for such substituted thyronines as triiodo thyronine which are less strongly associated with TBC_{EC} than thy roxine a more rapid rate of disappearance from the plasma might be anticipated even if their cellular k_3 were the same as that of thyroxine

Thus it would appear that if D represents the integrated product of at least two distinct processes cellular uptake and the fractional rate of cellular degradation then both processes must be considered in any interpretation of manifest rates of hormone removal from the thyroxine space Furthermore as outlined previously (p 540) these processes may be subject to interdependent as well as independent variation For example prolonged reduction in the availability of hormone to the cell such as might occur when TBC_{EC} is increased could alter cellular metabolic activity and result in a retardation of k_3 Similarly it is conceivable that a sustained reduction in TBC_{EC} could increase cellular uptake sufficiently to accelerate k as an even tual secondary consequence Alternatively the same intracellular processes that govern k_3 could condition the circulating concentrations of extracellular thyroxine binding components by affecting their intra cellular rates of synthesis and degradation

Finally some attention should be focused upon the metabolism of the binding components *per se* *In vivo* the cellular and extracellular thyroxine binding moieties are probably undergoing constant renewal and hence they cannot be viewed merely in static and passive dimen sions Indeed their metabolic fate could affect the evolution of D if (1) TBC_C T_4 is degraded as an intact unit or (2) TBC_{EC} T_4 enters the cell as an intact unit or (3) T_4 is made available to the cell as a consequence of the catabolism of TBC_{EC} at the cell surface The possi bility that TBC_C consists of intact or partially disrupted components of TBC_{EC} that have crossed the cell barrier is also within the realm of speculative possibility As yet none of these avenues have been ex amined experimentally However the extraordinary differences in the distribution and turnover of tracer quantities of I^{131} labeled D and L thyroxine isomers (Rall *et al* 1953 Freinkel *et al* 1957) would diminish the likelihood that a major role in the formation of D need be assigned to the metabolic fate of the binding components

THE KINETICS OF DISPOSAL OF CIRCULATING HORMONE

In man I^{131} labeled thyroxine is uniformly mixed throughout its distribution space within 24 to 48 hours after its intravenous ad

bound thyroxine in the thyroxine space TBC_C T_4 *is relatively* small at any single moment (2) relatively large changes in TBC_C T_4 need not produce proportional alterations in the measured values for the thyroxine space and (3) if distribution of TBC_C is homogeneous then dilution of TBC_C T_4 by a non binding cellular mass would be sufficiently great as to make demonstration of TBC_C T_4 difficult Nonetheless since cellular uptake (i e the formation of TBC_C T_4) must logically precede cellular disposition of thyroxine (i e the evolution of D) and since uptake is conditioned by the quantitative interactions between T_4 and extracellular binding and cellular binding components [or in any single site by (T_4) (TBC_{EC}) and (TBC_C)] rate limiting roles in the formation of D *in vivo* must be assigned to the static concentration of all three variables Moreover as in all equilibria involving charged interactants the effects of changes in pH upon distribution characteristics must be recognized (Klotz and Walker 1947) In such a static equilibration it becomes merely a question of semantics whether uptake entails the accumulations of unbound T_4 or of T_4 which is complexed to the weakest component of the TBC_{EC} melange namely albumin It is even conceivable that the extracellular to cellular transitional form of T_4 is in the uncharged state and governed by local pH factors In any event if the rate of hormonal degradation by cellular processes (k_3) were instantaneous then indeed the evolution of D would be determined entirely by the binding interactions between T_4 TBC_{EC} and TBC_C

However as adjudged by the *in vitro* experiments in simple saline media k_3 does not seem to be instantaneous Rather fractional degradation appears to follow first order reaction kinetics and to affect a constant proportion of the available thyroxine per unit time Thus the rate constant for degradation k_3 must be viewed as a second rate limiting factor in the formation of D

It therefore follows that at any given level of cellular uptake the rate of disposition of a fraction of the uptake could determine the quantity of D evolved per unit time Alternatively at any fixed values for k_3 the magnitude of cellular uptake could condition the availability of substrate for the processes described by k_3 and so regulate the amount of D that is formed The latter thesis was advanced as early as 1952 (Recant and Riggs 1952) to explain the normal availability of thyroid hormone to the tissues in conditions such as nephrosis wherein the extracellular thyroxine binding components

The TDS may be defined as the virtual volume of the body fluids in which the total quantity of exchangeable (extrathyroid) thyroxine would be distributed were it *present throughout at the same concentration* as that which occurs in the plasma It will be recognized that in actuality the concentration of thyroxine varies greatly among the plasma extravascular and cellular compartments Nevertheless it is possible to define TDS as follows

TDS = total extrathyroid thyroxine/concentration of thyroxine in plasma

$$= \frac{[(T_{4EC}) + (TBC_{EC}\ T_4)]\ V_{EC} + [(T_{4C}) + (TBC_C\ T_4)]\ V_C}{(T_{4P}) + (TBC_P\ T_4)}$$

As defined k_1 and k_2 are merely equilibratory constants for free thyroxine and extracellular and cellular binding proteins respectively while k_3 represents the rate constant for the degradation of thyroxine within the cell Both *in vitro* and *in vivo* studies indicate that the rate of distribution of thyroxine is much more rapid than its rate of degradation *In vivo* therefore k_1 and k_2 are probably not manifest once injected isotopic hormone is uniformly distributed through its space Further the fractional rate of turnover of hormone *in vivo* (K) may be considered a direct reflection of k_3 although the two rate constants are not numerically equal

The total quantity of hormone removed from the space per unit time D may then be represented as

$$D = \text{total extrathyroid thyroxine} \times K$$
$$D = TDS[(T_{4P}) + (TBC_P\ T_4)]\,K$$

However, $D = \text{cellular thyroxine} \times k_3$

Therefore *total extrathyroid thyroxine* $\times K =$ *cellular thyroxine* $\times k_3$

$$\frac{K}{k_3} = \frac{\text{cellular thyroxine}}{\text{total extrathyroid thyroxine}}$$

Cellular thyroxine space = cellular thryoxine/plasma concentration of thyroxine

$$\frac{K}{k_3} = \frac{\text{cellular thyroxine space}}{TDS}$$

It is thus apparent that the over all fractional rate of turnover of hormone within the thyroxine space bears a fixed relationship to the *fractional rate of cellular degradation This relationship* is dependent upon the proportion of total exchangeable hormone localized within the cell i e upon the proportion of the total thyroxine space con

ministration (Ingbar and Freinkel 1955) Beyond this interval calculated values for the volume of distribution of the injected material remain constant The curve depicting the subsequent variation with time of the concentration of labeled hormone in plasma is exponential in nature and is thought to represent net removal of hormone from within its distribution space Thus employing the isotope dilution principle repeated sampling of plasma following the administration of labeled hormone affords a means of assessing volume of distribution and peripheral turnover Refinements of this technique reduce the error introduced by failure of the injected material to equilibrate instantaneously and provide additional information concerning specific pathways of hormonal disposal These methods have been employed to study the kinetics of hormonal metabolism in normal subjects and in patients with a number of thyroid and nonthyroid disorders (Rawson *et al* 1953 Hamolsky *et al* 1953 Sterling *et al* 1954 Ingbar and Freinkel 1955 Sterling and Chodos 1956) Similar results have also been obtained by observing the metabolic fate of endogenously synthesized labeled hormone (Berson and Yalow 1954) however this technique is technically more difficult and has therefore been less widely applied

Before describing the results obtained with these procedures it seems advisable to define the parameters measured more clearly and to discuss their physiological significance ($TBC_{EC}T_4$) and (TBC_CT_4) have been defined as the concentration of thyroxine associated with extracellular and cellular thyroxine binding components respectively and (T_4) as the concentration of thyroxine not associated with binding components For the purposes of the present discussion it is necessary to introduce several additional definitions

(T_{4P}) = concentration of unbound thyroxine in plasma
(T_{4C}) = concentration of unbound thyroxine within the cell
(T_{4EC}) = concentration of unbound thyroxine in extracellular fluids
(TBC_PT_4) = concentration of thyroxine bound to protein in the plasma
V_P = plasma volume
V_{EC} = volume of extracellular fluids through which thyroxine is distributed
V_C = volume of cellular fluids through which thyroxine is distributed
TDS = total thyroxine distribution space
K = fractional rate of disappearance of thyroxine from the TDS

metabolic action Thus when abnormalities in the fractional rate of turnover of hormone are found during *in vivo* tests it is not possible to assess whether such abnormalities are generalized or localized to specific organs whether they represent disturbances in purely detoxifying or degradative reactions or whether they indicate a change in the stoichiometry of thyroid hormone action

It is against the background of these considerations that the discussion which follows immediately below as well as that which appears elsewhere herein concerning the kinetics of hormonal metabolism in normal subjects and in patients with various disease states should be projected

Normal Values

In normal adults the thyroxine binding capacity of TBG averages about 22 μg and that of TBPA about 120 μg per hundred milliliters of serum (Robbins and Rall 1957 Ingbar 1959) At essentially endogenous concentrations of PBI (i e as indicated by the addition of very small quantities of I^{131} labeled thyroxine) approximately 45 per cent of the circulating hormone is bound to TBG 40 per cent to TBPA and 15 per cent to albumin (Ingbar 1959) *In vivo* tracer doses of labeled thyroxine distribute through a volume of about 10 liters or 15 per cent of the body weight The fractional rate of peripheral turnover averages about 10 per cent per day so that half of the extrathyroid hormone is degraded and replaced each 6 to 7 days (thyroxine half time) Thus with an average normal PBI of 5 0 μg per hundred milliliters total exchangeable hormone will contain approximately 500 μg of iodine and of this 50 μg of hormonal iodine will be degraded daily This will be equivalent to the quantity of hormone contained in about 1 liter of plasma (10 liter × 10 per cent per day = 1 0 liter per day) volume turnover (Ingbar and Freinkel 1955 Sterling *et al* 1954)

Hyperthyroidism

In patients with active hyperthyroidism binding capacity of TBG is normal or very slightly increased while the binding capacity of TBPA may be normal but is usually diminished (Robbins and Rall 1957 Ingbar 1959) Concomitantly the concentration of circulating hormone is characteristically increased As a result, normal thyroxine binding equilibria are disturbed A lower than normal percentage of circulating hormone is associated with primary carriers (TBG and

tributed by its cellular component It therefore follows that disturb ances in hormonal binding equilibria which alter the distribution of thyroxine between cellular and extracellular confines and which therefore induce shifts in proportion of the cellular and extracellular thyroxine spaces will result in changes in the fractional rate of turn over of hormone as measured *in vivo* even when k_3 remains *unchanged*

Isotopic studies in man or animals have indicated that thyroxine *in vivo* is localized in the plasma extracellular and cellular fluids (Lipner *et al* 1952 Alpers and Rall 1955) and to this extent have confirmed the concept of a thyroxine space Quantitative studies *indicate that only a relatively small proportion of extrathyroid* hormone is localized in the cellular compartment (van Arsdel *et al* 1954) The extracellular cellular equilibrium for thyroxine thus seems to favor the extracellular phase Therefore the contribution of the cellular component to the total thyroxine space may be very small It is evident then that relatively large changes in the cellular portion of the space such as may be induced by alterations in binding equilibria may be manifested by only a small change in the total thyroxine space Changes of this magnitude may be difficult to perceive *in vivo* by the methods just described Similarly in the absence of changes in k_3 shifts in cellular extracellular equilibria favoring the cellular phase may result in only small increases in total hormonal degradation

However the *metabolic significance* of the cellular component need not be minimized since it is cellular hormone which is situated so as to be both metabolically active and metabolically altered As noted above shifts in cellular extracellular equilibria *will* alter K even if k_3 remains constant Furthermore it has already been suggested that alterations in the quantity of cellular hormone may induce either as an adaptive reaction or as a result of hormonal action an increase in the rate of the degradative processes that are reflected in k_3

Finally it should be noted that k_3 is an integrated rate constant reflecting degradative activity of multiple tissues whose individual rates of degradation may vary markedly *Furthermore neither* k_3 as presented in the hypothetical formulation nor the fractional rate of turnover of hormone as measured *in vivo* (K) differentiates between purely degradative or detoxifying metabolism of hormone and meta bolic alterations of the hormone incident to or necessary for its

is generally increased (Robbins and Rall 1957) Thus TBG is relatively undersaturated and secondary carriers such as albumin are called upon to transport less than the normal quantity of hormones In patients with severe myxedema the binding capacity of TBPA may be decreased (Ingbar 1959) This tends to restore the distribution of hormone between weakly binding secondary carriers (albumin) and strongly binding primary carriers (TBG in this instance) toward *normal Usually however at endogenous concentrations of hormone* the quantity of thyroxine transported by albumin remains subnormal In association with but not necessarily as a result of these alterations in binding equilibria fractional peripheral turnover of thyroxine is slowed Some contraction of the thyroxine distribution space is also evident These changes together with the subnormal PBI result in total degradation rates far less than the normal value Following restoration of eumetabolism by desiccated thyroid or thyroxine binding *equilibria and peripheral metabolism of hormone are restored to* normal (Ingbar and Freinkel 1955 Sterling and Chodos 1956)

ABNORMALITIES OF THE THYROID HORMONE–PLASMA PROTEIN COMPLEX

The foregoing sections have been concerned with the synthesis chemical properties and metabolic fate of the iodinated amino acids *which normally appear in the blood and with some of the properties* of the proteins in plasma with which they interact An earlier section has also described those thyroid disorders in which the abnormality is reflected in the blood primarily by an alteration in the concentration of hormone Attention is now directed toward those states in which the morbid process is accompanied either by the appearance of unusual iodinated moieties in the circulation or by alterations in the concentration or nature of the thyroxine binding proteins

Abnormal Iodinated Compounds in the Blood

Iodinated compounds normally foreign to the circulation may include *(1) those which are normal precursors of the circulating hormone* but which do not ordinarily find their way into the blood in appreciable quantities and (2) those which do not participate in the normal sequential synthesis of thyroid hormone For the latter nothing is known of the rates of peripheral disposal However for the former peripheral disposal is exceedingly rapid Since the momentary con

TBPA) while an abnormally high percentage is associated with the secondary carrier albumin While the thyroxine distribution space *in vivo* is indistinguishable from normal the fractional turnover rate is usually moderately to markedly increased and thyroxine half time diminished Increases in both volume turnover and PBI result in total degradatory rates far in excess of normal (Berson and Yalow 1954 Ingbar and Freinkel 1955 Sterling and Chodos 1956) However *acceleration of fractional turnover of thyroxine is not an invariable* finding in patients with active thyrotoxicosis Several patients have been observed whose turnover rates were well within the normal range Significant clinical differences between these patients and those with accelerated turnover rates could not be distinguished Elucidation of the cause of this divergence remains an intriguing challenge

Following restoration of a eumetabolic state a significant proportion of patients with Graves disease retain an abnormally rapid peripheral turnover of thyroxine which persists for varying periods of time having been observed for as long as six years after alleviation of active thyrotoxicity This acceleration of fractional turnover rate is not associated with abnormalities in either the binding or the concentration of the circulating thyroid hormone Rather it appears to arise in some intracellular focus (Ingbar and Freinkel 1958b) It also seems noteworthy that a comparable abnormality in the peripheral metabolism of thyroxine has been described in a number of relatives of patients with Graves disease who have not themselves been thyrotoxic (Ingbar *et al* 1956)

Hypermetabolic States

Thyrotoxicosis factitia whether induced by desiccated thyroid or by triiodothyronine and therefore whether associated with increase or *decrease in the circulating PBI is accompanied by acceleration of the* fractional rate of turnover of thyroxine (Ingbar and Freinkel 1958b) Similarly accelerated turnover rates are seen in patients given drugs which induce hypermetabolism such as salicylates (Austen *et al* 1958) In this case no alteration in the binding characteristics of TBG is induced

Myxedema

In patients with either primary or pituitary myxedema *distribution* of circulating thyroxine among the plasma proteins is distinctly altered Not only is the PBI low but the binding capacity of TBG

rally increased (Robbins and Rall 1957) Thus TBG is rela undersaturated and secondary carriers such as albumin are upon to transport less than the normal quantity of hormones ents with severe myxedema the binding capacity of TBPA may reased (Ingbar 1959) This tends to restore the distribution of ne *between weakly binding secondary carriers (albumin) and* y binding primary carriers (TBG in this instance) toward Usually however at endogenous concentrations of hormone intity of thyroxine transported by albumin remains subnormal ciation with but not necessarily as a result of these alterations ding equilibria fractional peripheral turnover of thyroxine is Some contraction of the thyroxine distribution space is also t These changes together with the subnormal PBI result in degradation rates far less than the normal value Following tion of eumetabolism by desiccated thyroid or thyroxine bind uilibria and peripheral metabolism of hormone are restored to l (Ingbar and Freinkel 1955 *Sterling and Chodos 1956)*

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inated compounds normally foreign to the circulation may in (1) those which are normal precursors of the circulating hormone which do not ordinarily find their way into the blood in ap ible quantities and (2) those which do not participate in the al sequential synthesis of thyroid hormone For the latter nothing own of the rates of peripheral disposal However for the former heral disposal is exceedingly rapid Since the momentary con

centration of a substance in the blood is the resultant of its rates of entry and of removal it is evident that presence in the blood of normal hormonal precursors may represent merely quantitative rather than qualitative disturbances in normal mechanisms leading to increased entry decreased disposal or both

Iodotyrosines in the Blood The kinetics of the peripheral metabolism of the iodotyrosines in normal individuals indicates that these compounds would be demonstrable in the circulation only if their rates of entry were markedly increased or their rates of removal greatly retarded Although few studies of the peripheral turnover of L mono iodotyrosine (MIT) have been performed in normal subjects the data are sufficient to indicate that this compound is very rapidly cleared from the plasma (Stanbury *et al* 1956a) Following a single injection of I^{131} labeled monoiodotyrosine (MIT) virtually all identifiable MIT has disappeared from the plasma within 4 hours and the plasma inorganic I^{131} has markedly increased Concomitantly a large fraction of the administered tracer has been excreted in the urine almost entirely as inorganic iodide Small amounts of MIT may be metabolized by non deiodinative pathways In normal individuals given labeled MIT there appears in serum a compound which migrates neither as MIT nor as inorganic iodide during chromatography in the butanol acetic acid system This chromatographic band is intensified in patients whose deiodinative pathways are impaired as will be discussed below

Similar studies in normal individuals given L diiodotyrosine (DIT) indicate that this too is cleared from the plasma very rapidly (Albert and Keating 1951 Stanbury *et al* 1956a) A small percentage (normally not more than 6 per cent) of the administered compound is excreted intact into the urine The remainder appears as inorganic iodide Intact DIT has virtually disappeared from the urine at the end of 4 hours Studies of the serum correlated well with urinary findings in revealing a very rapid rate of removal After several hours there remains in the serum less than 1 per cent of the administered dose per liter

It is tempting to associate the rapid disappearance of MIT and DIT from the circulation with their protein binding characteristics They are apparently completely devoid of association with TBG (Robbins and Rall 1955 Larson and Albright 1955) and only loosely bound to other proteins (presumably albumin) since they are only partially precipitable with the plasma proteins (Blizzard and Mosier 1957)

In view of the foregoing considerations it is not surprising that the great majority of workers have failed to find MIT or DIT in the circulation of euthyroid subjects following administration of either tracer or therapeutic quantities of I^{131} (Taurog and Chaikoff 1948 Laidlaw 1949 Rosenberg 1951 Dingledine *et al* 1955) In normal man close agreement between concomitantly estimated values for the daily degradation of thyroxine like compounds and the daily thyroid incorporation of iodine suggests that little besides thyroxine is released from the gland (Ingbar and Freinkel 1955) However kinetic evidence has been presented which suggests that compounds similar to the iodotyrosines may be released into the circulation in relatively large quantities in patients with thyrotoxicosis both before and during antithyroid therapy (Ingbar and Freinkel 1955 1958b) In one study seemingly significant quantities of these compounds have been found in the blood of patients with Graves disease (Benua and Dobyns 1955)

In 1953 and 1955 there appeared the first reports of patients in whom following administration of inorganic I^{131} large proportions of the radioiodine in serum were comprised of MIT and DIT The earlier report described the occurrence of DIT in the sera of two patients with seemingly endemic cretinism (Costa *et al* 1953) The second and subsequent reports described a family of congenitally goitrous hypothyroid individuals in whom studies served to delineate a new variety of thyroid disease (Stanbury *et al* 1955b 1956b) Such patients demonstrate an accelerated thyroid uptake of inorganic I^{131} associated with a rapid initial decline in the concentration of radioiodine in the plasma Organification of accumulated I^{131} is apparently unhindered since administration of thiocyanate does not result in appreciable immediate loss of glandular radioiodine Secondarily there occurs a rapid but unsustained decline in glandular radioactivity and during this period serum I^{131} rapidly rises to high levels At this time the radioactivity in serum is largely nonprecipitable Subsequently there occurs a phase of more prolonged release of glandular radioiodine and serum I^{131} becomes almost entirely precipitable Chromatograms of serum and urinary radioiodine in such patients reveal MIT DIT iodide T_4 and T_3 It has been suggested that during the phase of rapid glandular release the poorly precipitable MIT and DIT are the principal components released during the phase of more prolonged release the glandular product may be principally iodothyronines Similar abnormalities in the kinetics of iodine metab

olism had been reported earlier in a group of patients with cretinism or juvenile myxedema In these subjects release of diiodotyrosine was postulated but not demonstrated (Hubble 1953 Hutchison and McGirr 1954)

In such patients the metabolism of I^{131} labeled racemic MIT is strikingly abnormal Urinary iodine is almost entirely in the organic form In butanol acetic acid chromatograms of urine four radioactive bands are observed These include MIT iodide and two unidentified bands one of which lies between the origin and the iodide zone and corresponds in position to that faintly seen in normal individuals given MIT This unknown compound appears to be a conjugate of MIT since it yields MIT after gentle acid hydrolysis Following the administration of racemic DIT unchanged DIT persists in the serum for prolonged periods and constitutes the major urinary excretion product Finally in contradistinction to the dehalogenation of DIT and MIT which occurs during incubation with normal thyroid tissue little or no dehalogenation of these amino acids is effected by thyroid tissue from these patients (Querido *et al* 1956)

It has been postulated that there exists in such patients a genetic ally conditioned lack of dehalogenase activity for the iodotyrosines both in peripheral tissues and in the thyroid gland As a consequence large quantities of MIT and DIT are released into the circulation and lost in the urine Normally iodine evolved during the deiodination of MIT and DIT is available to the thyroid gland for reincorporation into hormonal moieties A conditioned iodine deficiency resulting in this manner may lead to hypothyroidism despite the manifest ability of the thyroid gland to synthesize and secrete T_4 and T_3

Recently another patient has been reported in whom the radio iodine in the serum following the administration of inorganic I^{131} was found to be comprised of MIT DIT T_4 and T_3 and iodine (Werner *et al* 1957a) In contradistinction to the patients described above however this mildly hypothyroid young girl demonstrated no apparent abnormality in the peripheral deiodination of DIT nor could an impairment of glandular dehalogenase activity be demon strated It was suggested that a defect in the intrathyroid coupling of iodotyrosines to form iodothyronines may have been present in this patient A similar defect has been proposed to explain the pathogenesis of goitrous cretinism in two patients previously reported (Stanbury and Querido 1956) but in these patients iodothyronines were apparently not demonstrated in the circulation (Stanbury *et al* 1955a)

At present evidence for a defect in coupling of iodotyrosines in patients such as these remains tenuous and the precise pathogenesis of the goitrous hypothyroidism remains obscure

Iodoproteins in the Blood

Thyroglobulin As noted earlier thyroglobulin and presumably other thyroid proteins are excluded from the circulation by the barrier of the follicular wall However it has recently been recognized that proteins of glandular origin may enter the circulation in a variety of abnormal situations Reconstruction of the pathogenesis of these disorders requires that the relationship of these proteins to thyro globulin be ascertained This task is complicated by recent observa tions which suggest that thyroglobulin itself may be a mixture of closely related proteins (Ingbar *et al* 1959) and that thyroid proteins clearly distinct from thyroglobulin may normally be iodinated within the gland (Robbins *et al* 1959) Thus a working definition of the term thyroglobulin becomes mandatory The authors proposed that this term be applied to iodinated protein of thyroid origin which demonstrates specific physicochemical properties These include (1) high molecular weight and rapid ultracentrifugal sedimentation (Heid elberger and Pedersen 1935 Derrien *et al* 1949) (2) specific solubility characteristics in salt solutions of high ionic strength (Derrien *et al* 1948) (3) isoelectric point of 4 5 (Heidelberger and Pedersen 1935) and (4) electrophoretic migration of pH 8 6 corresponding to the inter alpha zone of serum (Ingbar and Freinkel 1957) Although immuno logical cross reactivity between thyroglobulin and other thyroid pro teins may be invoked as a means of identification this criterion would seem less rigid than those just described since precursors of thyro globulin might demonstrate immunological cross reactivity Employing these criteria it is possible to categorize diseases in which iodoproteins of glandular origin appear in the circulation into those in which (1) the iodoprotein is thyroglobulin and is therefore a precursor of the circulating hormone and (2) the iodoprotein does not conform to a known stage of hormonal synthesis

Since thyroglobulin is rapidly cleared from the circulation detect able quantities should be present only when the rate of entry of the protein into the blood is rapid By means of immunological techniques thyroglobulin or proteins closely related thereto have been found in the thyroid venous effluent of patients undergoing thyroidectomy and in their general circulation for from 24 to 36 hours thereafter (Lerman

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1940) Here release of protein apparently results from mechanical or possibly anoxic trauma of the gland

Acute inflammatory lesions of the thyroid gland also make possible the escape into the circulation of thyroglobulin and related iodoproteins and iodopeptides During the acute thyroiditis which may follow the administration of therapeutic doses of I^{131} a moderate to large fraction of the radioiodine in the serum may be found in a form which is protein precipitable but not extractable with butanol This chemical evidence indicates that the radioiodine is not part of a molecule which is loosely adsorbed to the surface of a plasma protein but rather forms an integral portion of the protein molecule as is the case in thyroglobulin Frequently a marked decline in thyroid radioactivity accompanies the appearance of this iodoprotein in the blood and occasionally there occurs an increase in the concentration of PBI^{127} It has been further possible to demonstrate that this iodoprotein possesses the solubility and ultracentrifugal characteristics of thyroglobulin and upon hydrolysis yields iodide and iodinated amino acids in about the proportion in which they are found in intrathyroid thyroglobulin (Robbins *et al* 1952 Robbins 1954) The severity of this acute disruption of thyroid storage mechanisms seems related to the quantity of radiation delivered to the gland and to the severity of the resulting inflammatory response

Patients with subacute thyroiditis frequently demonstrate an increase in PBI^{127} in the plasma (Fraser and Harrison 1952) which may or may not be accompanied by an increase in basal metabolic rate together with a marked diminution in thyroid capacity to accumulate I^{131} (Werner *et al* 1949 Towery 1956) Recent studies have shed some light on this seeming paradox (Ingbar and Freinkel 1958a) By examination of radioiodinated moieties in the serum following tracer doses of I^{131} it has been shown that despite the profound reduction in thyroid accumulation of radioiodine which occurs in this disease some organic iodinations presumably thyroid in origin do occur Both butanol soluble and butanol insoluble compounds frequently comprise an abnormally large proportion of total precipitable I^{131} Similarly usually large discrepancies between the protein bound and butanol extractable I^{127} (PBI^{127} and BEI^{12}) may be seen Attempts at immunological identification of the circulating iodoproteins in patients with subacute thyroiditis have thus far failed It was postulated that destruction of follicular integrity as a consequence of the inflammatory lesion makes possible the escape into the thyroid

interstitium and the circulation of thyroglobulin and products of its hydrolysis.

Probably the most common disorder associated with the release into the blood of thyroglobulin or closely related proteins is struma lymphomatosa (Hashimoto's disease). It had been recognized for some time that patients with this disease could display hypometabolism or clinical myxedema despite the accumulation of normal or increased quantities of I^{131} in the thyroid gland (Furr and Crile, 1954). From this it could be inferred that the thyroid gland was producing materials of low calorigenic potency. Further evidence that this might be true was provided by the demonstration that in some patients with Hashimoto's disease, an abnormally large discrepancy exists between the concentration in serum of PBI^{127} and BEI^{127} (Gribetz *et al.*, 1954; Skillern *et al.*, 1956; Owen and McConahey, 1956). This finding was complemented by the additional observation that following tracer doses of radioiodine, an unusually large proportion of the PBI^{131} is not extractable with butanol (Owen and McConahey, 1956). These evidences for the release of thyroid protein into the circulation were climaxed by the demonstration that the hypergamma globulinemia noted in patients with this disease is associated with the presence in serum of precipitating or complement-fixing antibodies against saline extracts of thyroid gland or against purified thyroglobulin (Roitt *et al.*, 1956; Doniach and Roitt, 1957; White, 1957). These observations followed extensive work on thyroid autoimmunity in animals (Rose and Witebsky, 1956) and stimulated widespread interest in autoimmune mechanisms as they might apply to other diseases of the thyroid gland. At present, it seems clear that circulating antibodies against thyroglobulin may be found in a variety of thyroid diseases not generally considered to be related to Hashimoto's struma (Roitt and Doniach, 1958; Blizzard *et al.*, 1959). Thus the precise role of autoimmune mechanisms in the genesis of this or other thyroid diseases remains to be clarified. It is apparent, however, that no explanation has as yet been offered to account for the initial passage of thyroglobulin from its site of sequestration within the follicle to its site of antigenic action. However, one interesting aspect of the postulated pathogenetic sequence is the implication that release of unhydrolyzed thyroglobulin would deprive glandular dehalogenases of the opportunity to recycle iodine bound as MIT or DIT.

Non-thyroglobulin Iodoproteins. In contradistinction to the foregoing disorders in which thyroglobulin seems to escape from the thy-

roid gland there occur a number of situations in which iodoproteins clearly distinct from thyroglobulin have been found in the blood

In 1955 there appeared the first report of an abnormal iodinated protein in the blood of more than half of 23 patients with functioning carcinoma of the thyroid gland (Robbins *et al* 1955) In this and a subsequent report (Tata *et al* 1956) several characteristics of this protein have been elucidated This material designated compound X was found to be nondialyzable and heat coagulable Both the electrophoretic mobility of compound X in free and starch block electrophoresis and its sedimentation constant in serum were found to be similar to those of albumin Alcohol fractionation of the serum proteins could separate compound X from thyroglobulin but not from albumin However by virtue of its failure to react with anti albumin immune serum and its coagulation by heat in the presence of 0 02 molar sodium caprylate compound X seemed clearly not an iodinated albumin Upon hydrolysis compound X contained principally MIT A second component having the same chromatographic mobility as thyroxine was also seen but in the solvent system employed this compound could not be distinguished from 3 3 diiodothyronine

The chemical concentration of compound X in the serum of patients with carcinoma of the thyroid is unknown as is its rate of turnover Nor is it entirely clear what relationship if any compound X bears to thyroglobulin Preliminary experiments suggest that compound X may be precipitated by anti thyroglobulin antisera (Ingbar and Tata 1958) These findings would suggest that the two proteins are closely related Of interest in this regard is the possibility that release of compound X in patients with thyroid carcinoma might induce autoimmune responses in the thyroid gland analogous to those which occur in Hashimoto s disease

Abnormal circulating iodoproteins are apparently not found exclusively in patients with functioning thyroid carcinoma Recently reports of 4 other patients with unusual iodoproteins in the blood have appeared (Whitelaw *et al* 1956 DiGeorge and Paschkis 1957 Werner *et al* 1957a DeGroot *et al* 1958) These have been studied by varying techniques so that it is difficult to ascertain whether or not they represent the same pathophysiological entity All of the 4 patients were hypometabolic but only 2 were goitrous Thyroid uptakes were usually increased and butanol nonextractable I^{131} was found in abnormally large concentrations in the sera In one instance of particular interest an iodoprotein with the electrophoretic and ultracentrifugal charac

teristics of compound X was found in the serum of a congenitally goitrous hypothyroid patient (DeGroot *et al* 1958) Recent studies in the authors laboratory have revealed the presence of a similar iodo protein in the serum of several patients with nontoxic goiter appar ently arising during adult life These patients demonstrated increased thyroid uptake of I^{131} During zone electrophoresis of serum a large proportion of protein bound radioiodine migrated in the albumin area and this fraction could not be extracted with butanol

It seems evident as increased interest is directed toward this area that disorders of protein synthesis or storage may prove to be relatively common pathophysiologic lesions in patients with nontoxic goiter

ABNORMALITIES OF THE THYROXINE-BINDING PROTEINS

The possible contribution of thyroxine binding interactions to thyroid economy has been outlined in a foregoing section If regulatory functions are indeed mediated by protein binding phenom ena alteration in these interactions should be associated with disturb ances in the metabolism of iodine A search among various clinical states for changes in the activity of the thyroxine binding proteins might therefore serve a twofold purpose First consistent association of changes in hormonal binding with specific alterations in hormonal economy might serve to elucidate the physiological effects of the binding protein Second some light might be shed upon disease states demonstrating otherwise unexplained changes in the metabolism of iodine The following section will describe those situations in which as a result of this search binding interactions have been shown to be abnormal

Abnormalities of Thyroxine binding Globulin

Normal Pregnancy and Effects of Estrogen Pregnancy is accom panied by a variety of well documented but little understood altera tions in thyroid morphology and in the metabolism of iodine These include thyroid hyperplasia and occasionally gross thyromegaly (East man 1950) together with increased thyroidal uptake of I^{131} and in creased turnover of glandular iodine (Pochin 1952 Ferraris and Scorta 1955) In addition during normal pregnancy there occurs an increase of about 3 μg per hundred milliliters in the concentration of circulat ing protein precipitable and butanol-extractable iodine (Heinemann

et al 1948 Russell 1954) The latter change begins about three weeks after conception persists throughout gestation and gradually recedes after delivery During normal pregnancy this complex of findings which is ordinarily associated with thyrotoxicosis is not accompanied by symptoms of thyrotoxicity or by an increase in the basal metabolic rate * Because of this dichotomy thyroxine protein interactions in the serum of pregnant women were among the earliest to be subjected systematic scrutiny Studies in normally pregnant women revealed a marked increase in thyroxine binding by TBG as evidenced by findings obtained during paper electrophoresis in Veronal buffer at pH 8 6 (Dowling *et al* 1956c) This change was often appreciable as early as the twenty fourth post ovulatory day increased during the next several weeks remained constant throughout the duration of gestation and then gradually declined during the ensuing 4 to 6 weeks (Dowling 1956c) Qualitatively the TBG of pregnancy could not be distinguished from the TBG of normal serum In addition abnormally avid binding of thyroxine by TBG was demonstrated when the proteins in sera from pregnant subjects were diluted to their approximate concentration in the interstitial fluid This is the concentration of protein with which cellular sites for the accumulation of thyroxine are presumably interacting Similar though less marked changes in thyroxine binding by TBG were found in umbilical cord blood Subsequently by means of the reverse flow technique these findings were confirmed and quantitative dimensions for the alteration in thyroxine binding were fixed (Robbins and Nelson 1958) The saturation capacity of TBG in serum was found to increase two to threefold during normal pregnancy Preliminary studies in the tris maleate system suggest that thyroxine binding by TBPA changes little if at all during normal pregnancy may decrease during labor and declines abruptly in the immediate post partum period In this buffer system the quantitative changes in the binding capacity of TBG demonstrated by the reverse flow system have been confirmed (Ingbar 1959)

Despite these demonstrations of yet another abnormal aspect of thyroid economy during pregnancy an integrated hypothesis concerning the causes of these abnormalities and their peripheral effects is still lacking It seems likely that changes in thyroxine binding by TBG are responsible for the marked reduction in red blood cell

* Although the BMR normally increases during the latter half of pregnancy this increase has been ascribed by some to the metabolic needs of the fetus (Sandiford and Wheeler 1924)

uptake *in vitro* of I^{131} labeled thyroxine and triiodothyronine from the sera of pregnant women (Hamolsky *et al* 1957b) Similarly the relatively rapid increase in erythrocyte uptake to normal values following delivery seems consistent with the abrupt decline in the binding avidity of TBPA which occurs post partum (Ingbar 1959) Nevertheless it is difficult at present to correlate these *in vitro* observations with phenomena occurring *in vivo* Although the concentration of circulating thyroid hormone in the pregnant woman is readily determined reluctance to administer radioactive isotopes to these patients has prevented the determination of such critical parameters as the volume of distribution of hormone and its fractional rate of turnover These data are necessary for estimation of the total quantity of hormone degraded

Since both synthetic and naturally occurring estrogens are capable of reproducing in nonpregnant individuals an increase in both PBI (Engstrom and Markardt 1954) and TBG comparable to those which occur during pregnancy (Dowling *et al* 1956b) it seemed possible that evidence relating to the physiological role of TBG and to the kinetics of thyroxine metabolism in pregnancy might be obtained in patients treated with estrogens Accordingly the effects of both synthetic and naturally occurring estrogens on the distribution and peripheral turnover of tracer doses of I^{131} labeled thyroxine have recently been studied (Dowling *et al* 1958) During the period of estrogen action there occurred a consistent reduction in the fractional rate of turnover of thyroxine together with a small but significant increase in its volume of distribution Increases in PBI were induced in both normal subjects and patients with treated myxedema This finding in the latter group confirms the slowing of peripheral turnover indicated by studies with labeled hormone Despite the reduction in the fractional rate of turnover of thyroxine induced by estrogen the total quantity of hormone degraded daily did not change since the extra thyroid pool of hormone concomitantly increased (enlarged thyroxine space and increased PBI) Attempts to dissociate temporarily the genital effects of estrogen (as evaluated by the vaginal smear) from the effects of estrogen on TBG and the turnover of thyroxine were unsuccessful It is thus uncertain whether the slowing of thyroxine turnover observed in these studies was the result of increased TBG or of a direct cellular effect of the estrogenic hormones

There are of course profound differences between the pregnant patient and the patient receiving estrogenic hormones First the

estrogenic hormones employed do not induce an increase in thyroid I^{131} uptake analogous to that seen in the pregnant woman (Engstrom and Markardt 1954 Dowling *et al* 1956b) Secondly peripheral tissues in the pregnant woman are subjected to the metabolic impact of large quantities of hormones other than estrogens Finally the conceptus itself may profoundly contribute to the over all distribution and destruction of thyroxine For these reasons direct extrapolation to pregnant individuals of results obtained in patients given estrogens is not possible Nevertheless it seems possible that in the pregnant woman as in the patient receiving estrogen total degradation of thyroxine by tissues other than the uterus and its contents might be normal To the extent then that the pregnant uterus and the conceptus degrade thyroxine total body degradation would exceed the normal value

The view that total body degradation of thyroxine is greater than normal in the pregnant woman is consistent with earlier demonstrations that the thyroid uptake and turnover of radioiodine are increased during pregnancy (Pochin 1952 Ferraris and Scorta 1955) and that the radioiodinated products appearing in the blood are the active butanol extractable hormones thyroxine and triiodothyronine (Ferraris and Scorta 1955) Confirmatory evidence has been obtained in patients with hydatidiform moles (Dowling *et al* 1959) Like normally pregnant women these patients demonstrate increased PBI TBG and thyroid uptake of I^{131} Estimated values for the daily thyroid accumulation of stable iodine calculated from the specific radioactivity of the urinary I^{131} have been found to be markedly increased

It is evident that an increase in the total delivery of hormone from the thyroid gland could be accomplished in the pregnant woman without the intercession of an increase in TBG or PBI The function if any of these alterations therefore remains unclear Increases in TBG may serve to limit the transplacental passage of thyroid hormone thereby minimizing the oxidative requirements of fetal tissues and additionally providing a stimulus to the function of the fetal gland These and other fascinating aspects of the hormonology of pregnancy await direct experimental elucidation

Abnormal Pregnancy In the initial publication fully describing alterations in TBG during normal pregnancy data were presented which had been obtained in 8 patients undergoing spontaneous abortion (Dowling *et al* 1956c) In this group failure to develop the normal gravid increase in TBG was noted These findings have been

confirmed and extended in an additional 32 patients Where mechanical factors such as uterine leiomyomata can be excluded spontaneous abortions occurring up to the tenth or twelfth week of gestation were accompanied by negligible or subnormal increases in TBG Beyond the tenth or twelfth week of pregnancy fetal loss most often occurred despite full development of the usual gravid augmentation of TBG This observation together with the slow decline in the binding capacity of TBG following normal full term delivery suggests that when subnormal responses in TBG are seen in patients with early abortion they can be explained by failure of appearance rather than by rapid regression of the normal gravid response

In pregnancies terminating in early abortion subnormal increases in TBG have been found prior to the onset of symptoms of abortion and at a time when tests for chorionic gonadotropin in the urine were positive Conversely demonstration of normal gravid changes in TBG has been employed as a good prognostic index to avert dilation and curettage in women who have subsequently gone to term following a threatened abortion early in pregnancy In addition many patients threatening to abort may display slight to moderate though still subnormal increases in TBG after tests for urinary gonadotropin have become negative It has therefore been possible to distinguish threatened abortion from dysfunctional uterine bleeding and ectopic pregnancy from other lower abdominal disease Similarly prolonged persistence of the TBG changes of pregnancy has been found in patients with retained secundines or with molar degeneration of placental remnants (Ingbar *et al* 1958)

In early abortion as in pregnancy the concentration of circulating thyroid hormone and the binding capacity of TBG appear to correlate closely Inadequate PBI response to pregnancy in aborting women (Heinemann *et al* 1948) has been shown in the foregoing studies to be associated with failure of TBG to increase to normal pregnancy values (Ingbar *et al* 1958)

Uncertainty concerning the physiological role of the augmented TBG and PBI in normal pregnancy makes hypotheses concerning the participation of inadequate TBG and PBI response in the genesis of abortion entirely speculative Since the response of both can be induced by estrogens it seems likely that in most instances of spontaneous abortion failure to increase TBG to a normal degree reflects inadequate secretion of estrogen probably secondary to some abnormality in the conceptus itself However to the extent that the increase in

PBI and TBG subserve some useful function their inadequacy under such circumstances may contribute to ultimate fetal wastage

Effects of Androgen Androgenic hormones apparently produce changes in the metabolism of thyroxine which are the direct converse of those induced by estrogens Recent data indicate that androgenic hormones may induce (1) decreased PBI (Keitel and Sherer 1957) (2) decreased binding capacity of TBP (3) increased fractional rate of turnover of thyroxine peripherally and (4) no change in the total quantity of thyroxine degraded daily (Federman *et al* 1958) It is necessary to emphasize however that in the case of both androgens and estrogens pharmacological doses of hormone are required to produce effects No significant difference is demonstrable in the PBI TBG fractional turnover rate and daily degradation rate for normal men and women

Nephrosis Like pregnancy the nephrotic syndrome presents perplexing evidences of alterations in thyroid economy Although hypometabolism and hypercholesterolemia frequently accompany the nephrotic state they are not in themselves pathognomonic of thyroid disorder Rather it is the frequent occurrence of a decreased plasma PBI which bespeaks a disturbance in the metabolism of iodine (Peters and Man 1948) This decrease in PBI associated with severe hypoproteinemia and proteinuria has led to the hypothesis that in nephrosis considerable quantities of thyroid hormone might be lost into the urine either free of or in association with plasma protein Efforts to assess this hypothesis have led to conclusions which are virtually diametrically opposed In an early study (Recant and Riggs 1952) it was found that in nephrosis thyroid accumulation of I^{131} was normal or increased and that the thyroid gland was capable of response to exogenous TSH Loss of hormone in the urine though substantial was not considered adequate to account for the low PBI It was concluded that in nephrosis the supply of hormone to peripheral tissues is probably adequate and hence the hypometabolism and increased plasma cholesterol arise from nonthyroid causes However results of a subsequent study (Rasmussen 1956) indicate that because of pronounced urinary and fecal losses of hormone the supply of hormone available to the peripheral tissues in patients with nephrosis is probably diminished Thus peripheral hypothyroidism might indeed exist Failure of the thyroid gland to respond to this seeming stimulus was ascribed to possible alterations in hypophyseal function resulting from inani

tion or protein loss At present it is not possible to reconcile these conflicting kinetic data

Divergent data concerning thyroxine protein interactions in the sera of nephrotic patients have also been reported (Recant 1956 Robbins *et al* 1957) These apparent discrepancies have probably arisen from methodological differences Results obtained with the more reliable reverse flow technique using barbital buffer indicate that thyroxine added to nephrotic sera binds to an inter alpha migrating protein similar to TBG in its physicochemical properties (Robbins *et al* 1957) Albumin acts as a secondary carrier for thyroxine when the concentration of hormone in the sample is enriched Of some interest is the observation that a pre albumin occasionally appears as a prominent thyroxine binding component in nephrotic sera even when electrophoresis is performed in Veronal buffer TBG has similarly been found in the urine and ascitic fluid of nephrotic patients although its presence in the urine is not invariable A small group of sera and urines from nephrotic patients have recently been studied in the authors laboratory by electrophoresis in the tris maleate buffer system These preliminary data reveal that the thyroxine binding capacity of TBG and to a lesser extent TBPA may be diminished in the serum While TBG was present in each of the five urines examined pre albumin was found in only one In several instances the binding capacity of TBG per gram of urinary protein was considerably greater in the urine than the serum These findings correlate well with the earlier observation that in this disease iodine/protein ratios often exceed those found in the serum (Recant and Riggs 1952) and that the *in vitro* uptake of labeled thyroid hormones from nephrotic sera may be abnormally high (Hamolsky *et al* 1959)

Liver Disease In man the quantitative importance of the liver in the degradation and excretion of thyroxine has not been entirely clarified An enterohepatic circulation of hormone of the magnitude found in rats seems unlikely (Albert and Keating 1952 Johnson and Beierwaltes 1953 Myant 1956) However alterations in hormonal economy which occur in diverse diseases of the hepatobiliary system indicate that this organ is significantly concerned in the metabolism of the thyroid hormone and its binding proteins Following the administration of I^{131} labeled thyroxine a considerable proportion of the administered material is quickly localized in the region of the liver and an equilibrium between hepatic and circulating hormone

is thereafter maintained The fate of the hormone thus localized is uncertain Quantitative studies indicate that only a small fraction of administered hormone is excreted in the bile reportedly as both intact thyroxine and its glucuronides (Scazziga *et al* 1955) and of these moieties apparently little is resorbed (Johnson and Beierwaltes 1953) The remaining portion of the hormone or its metabolic products which appears in the stool may result from direct passage into the lumen of the gastrointestinal tract

In patients with extrahepatic biliary obstruction glucuronides of thyroxine are said to appear in the blood (Vanotti 1957) but gross disturbances in over all hormonal economy have not been described

In contrast patients with acute hepatocellular disease such as infectious hepatitis are said to show striking alterations in the metabolism of iodine and of thyroid hormone During the acute state PBI levels are often increased to the thyrotoxic range (Kydd and Man 1951 Vanotti and Beraud 1959) while thyroid collection and turnover of I^{131} are decreased Augmented PBI levels are associated with increased binding of thyroxine by TBG and slowing of the peripheral turnover of hormone Hepatic conjugation of thyroxine is apparently decreased (Vanotti and Beraud 1959)

Comparably complex disturbances may also occur in patients with chronic hepatocellular disease PBI levels are normal or low The latter finding was initially correlated with the frequently coexisting hypoalbuminemia but in the light of current knowledge concerning the specificity of thyroxine binding this correlation would not seem to pertain On the other hand profound reductions in the binding capacity of TBPA are commonly found in patients with cirrhosis while an occasional patient may demonstrate an increase in binding by TBG perhaps as a result of defective metabolism of estrogenic steroids (Ingbar 1959) *In vitro* uptakes of labeled thyroid hormones by cellular systems are frequently increased (Hamolsky *et al* 1959) and glandular avidity for radioiodine is also often augmented (Mueller *et al* 1954)

The multiplicity of the synthetic degradatory and excretory functions of the liver virtually assures that interpretation of the foregoing fragmentary findings would be difficult *It is not possible at present to assess the extent to which these changes are caused by* abnormalities in the metabolism or excretion of the circulating hormone or its binding proteins or perhaps by metabolic abnormalities in extrahepatic tissues resulting from disease in the liver

Abnormalities in the Thyroxine binding Pre albumin

In the brief period which has elapsed since a technique was developed for the demonstration of thyroxine binding by TBPA in whole serum observations in the authors laboratory have revealed abnormalities in the binding of hormone by this protein in a number of disease states Initial observations indicated that the binding capacity of TBPA is moderately to markedly decreased in a large percentage of patients with untreated Graves disease On the other hand comparable reductions in thyroxine binding by TBPA were found in patients with severe myxedema In both groups findings returned to normal after restoration of a eumetabolic state Thyroxine binding by TBPA was also found to be decreased in serum obtained from patients 1 to 2 days post partum In view of these findings observations were extended to include sera obtained from patients with a wide variety of medical and surgical illnesses (Richards *et al* 1959a) Moderate to marked reductions in binding capacity were found in most but not all moderately or seriously sick patients Patients with hepatic cirrhosis or visceral malignancy most consistently demonstrated this abnormality However comparable reductions have been noted in many other disease states In general the degree of reduction in thyroxine binding by TBPA seems more clearly correlated with the severity rather than the nature of the illness

Serial studies in individual patients indicate that thyroxine binding by TBPA may decrease abruptly during the interval between 24 hours prior to and one day after delivery Similar studies have revealed precipitous declines in the binding activity of TBPA in normal subjects given typhoid vaccine or developing acute febrile illness The mechanisms whereby these decreases in thyroxine binding are mediated have not yet been clarified Conceivably they may represent an abrupt decrease in the concentration of the protein itself Alternatively it is possible that compounds other than thyroxine may be bound by TBPA These could increase in concentration in the plasma of the sick patient and might thereby interfere with the binding of thyroxine

The kinetic consequences of reductions in the thyroxine binding capacity of TBPA and the peripheral metabolism of the hormone are still unknown Recent studies have indicated that such reductions occurring in the face of unaltered concentrations of TBG may lead to marked increases in the *in vitro* uptake of thyroid hormones from

is thereafter maintained The fate of the hormone thus localized is uncertain Quantitative studies indicate that only a small fraction of administered hormone is excreted in the bile reportedly as both intact thyroxine and its glucuronides (Scazziga *et al* 1955) and of these moieties apparently little is resorbed (Johnson and Beierwaltes 1953) The remaining portion of the hormone or its metabolic prod ucts which appears in the stool may result from direct passage into the lumen of the gastrointestinal tract

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serum by particulate systems such as human erythrocytes Such decreases in the binding of thyroxine by TBPA have been proposed as being largely responsible for the high cellular uptake *in vitro* of thyroxine and triiodothyronine from the serum of thyrotoxic subjects since comparably high uptakes have occurred from the sera of patients with nonthyroid illness in whom binding by TBPA was decreased (Richards *et al* 1959a b)

Studies from several laboratories have revealed an abnormally rapid peripheral turnover of thyroxine in postoperative patients (Goldenberg *et al* 1957) in patients with leukemia or fever with associated hypermetabolism (Sterling and Chodos 1956) and in patients with visceral malignancy (Richards *et al* 1959b) It is tempting to attribute these alterations in the metabolism of thyroxine in sick patients to the changes in the binding of the hormone which occur under these circumstances However this suggestion is at best tentative Nevertheless these observations suggest that the thyroid gland may be significantly implicated in the metabolic response to acute and chronic illness

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CHAPTER XVI

Epinephrine and Norepinephrine

Marthe Vogt

SOURCES OF EPINEPHRINE AND NOREPINEPHRINE IN BLOOD AND CONDITIONS LEADING TO THEIR RELEASE

FATE OF CATECHOLAMINES IN THE BLOOD

ESTIMATION OF INDIVIDUAL CATECHOLAMINES IN BLOOD
- Choice of procedure
- Estimation using bioassay
  - Epinephrine
  - Norepinephrine
- Fluorimetric estimations

CONCLUSION

BIBLIOGRAPHY

SOURCES OF EPINEPHRINE AND NOREPINEPHRINE IN BLOOD AND CONDITIONS LEADING TO THEIR RELEASE

In contrast to other hormones present in peripheral blood, epinephrine and norepinephrine enter the blood stream nearly always as a result of nervous activity. Impulses traveling along the preganglionic cholinergic fibers of the splanchnic nerves release both amines from the adrenal medulla directly into the blood; stimuli passing along the postganglionic adrenergic fibers of the sympathetic system liberate sympathin, which consists essentially of norepinephrine, at the nerve terminals; this sympathin transmits the impulses from nerve ending to effector tissue and is partly metabolized *in situ*. In the process, a certain proportion, however, may leak into the circulation and contribute to the catecholamine level of the blood. This proportion is greatly enhanced by unphysiologically high rates of nerve stimulation or by antiepinephrine drugs of the dibenamine group (Brown and Gillespie, 1957). If the sympathetic division of the nervous system is completely at rest, blood levels of catecholamines should approxi-

mate zero It is not certain whether a very small basic secretion from the adrenal medulla does not persist after complete denervation in the cat traces of both amines were detected in adrenal vein blood of denervated adrenals (Vogt 1952a) representing about 0 5 to 1 0 per cent of the quantity normally released on electrical stimulation of the nerves It is impossible to decide whether this residual secretion represents a basic activity of the gland which is not of nervous origin or whether it is due to the fact that a few nerve fibers are liable to escape surgical denervation procedures There are also certain abnormal conditions mostly of an artificial nature which cause the release of medullary amines by direct stimulation of the chromaffin cells such as asphyxia arterial injections of potassium chloride and the administration of compounds which include naturally occurring substances like acetylcholine and histamine Asphyxia or high levels of blood acetylcholine in cases of poisoning with anticholinesterases might therefore produce medullary secretion of non nervous origin in a human patient (The direct effect of asphyxia on the adrenal medulla is superimposed on the much greater indirect effect due to stimulation of the sympathetic centers)

Epinephrine and norepinephrine also occur in the body in chromaffin tissue outside the adrenal medulla these so called paraganglia are particularly abundant near the abdominal aorta (organs of Zuckerkandl) but are also found in all sympathetic plexus and ganglia particularly in the large abdominal plexus It is not known whether chromaffin cells also occur in complete dissociation from the sympathetic system We have no knowledge of the role played by the amines in this extramedullary chromaffin tissue and do not know whether they ever enter the blood stream The paraganglia appear however to be innervated (Kohn 1903) and may have a greater physiological importance in infancy when they are large than in adult man in whom they are atrophic

The question is often asked what constitutes a normal level of blood epinephrine It will be obvious from the foregoing remarks that this question lacks the meaning it has in the case of other endocrine glands Blood levels normally reflect the activity of the sympathetic system and not the state of the adrenal medulla only when there is extensive destruction of medullary tissue or severe depletion of its amine stores will the condition of the medulla limit the level of epinephrine in the circulation Depletion is only encountered in very abnormal conditions because the stores of medullary hormones

are large and stimulation is rarely prolonged Experimentally deple tion can be produced by large doses of insulin and by repeated administration of stimulants of the sympathetic centers of acetyl choline or of reserpine In man subjected to repeated insulin shock or to prolonged therapy with reserpine such depletion is likely to take place It may also be present in cases of prolonged traumatic shock Resynthesis of medullary amines is a slow process (Arman 1951 Udenfriend *et al* 1953 Butterworth and Mann 1957) and once serious depletion has set in lack of preformed amines may limit secre tion for at least a number of days

Except then for these abnormal states of low hormonal stores blood levels of catecholamines vary with the state of activity of the individual (sleep and wakefulness) with temperament and with environmental circumstances which may favor or inhibit sympathetic activity

If one reason for wanting to know the blood levels of catecholamines is to obtain information on the activity of the autonomic nervous system the other is the hope of correlating certain clinical signs with the levels of epinephrine and norepinephrine in the blood Since the effects of intravenously administered epinephrine on pulse rate blood pressure diameter of skin vessels size of the pupil number of circu lating white blood cells etc are well known it is often assumed that such signs observed in a patient prove that his adrenal medulla is being stimulated Thus the tachycardia seen in the hypoglycemic reaction after insulin was for a long time attributed to release of epi nephrine till observations on patients who had been subjected to splanchnotomy and lumbar sympathectomy showed that the tachy cardia persisted while adrenomedullary secretion was no longer elicited by insulin (French and Kilpatrick 1955) The tachycardia is probably produced by stimulation of the nervi accelerantes and this usually does not lead to a raised level of catecholamines in the blood Gen erally speaking it is difficult to distinguish between the effects of stimulation of adrenergic nerves and of medullary secretion when ever the two are very similar Thus they are indistinguishable on coronary flow or heart rate they are different when gauged by the relaxation of certain vascular beds or of uterine muscle of some species The difference is of course accounted for by the predomi nance of epinephrine in medullary secretion and of norepinephrine in sympathin It is of some interest that epinephrine is 10 to 20 times more potent in its metabolic effects than norepinephrine and

that medullary secretion may affect metabolism under conditions when a rise in blood pressure is not produced

FATE OF CATECHOLAMINES IN THE BLOOD

When epinephrine is released into the circulation by the adrenal medulla, it is rapidly taken up by the tissues to which it is carried This is shown in model experiments in which the fate of injected epinephrine is followed by comparing the effects of injections of the amine into different arteries and veins (Dawes 1946 Celander and Mellander 1955) Some of the amine which is secreted into the plasma may attach itself to the red cells but it requires several hours incubation at 38°C to reach an equilibrium by which time the epinephrine is distributed approximately evenly between the two phases only that epinephrine present in the plasma is physiologically active (Bain *et al*, 1937)

The presence of catecholamines in platelets is affirmed by some and denied by other authors Weil Malherbe and Pone (1954b) described the occurrence of both epinephrine and norepinephrine in human platelets and explained the high values for amine concentrations they obtained in blood by this observation Born and Hornykiewicz (1957) found much epinephrine and no norepinephrine in pig platelets they further described an uptake of epinephrine and not of norepinephrine by pig platelets suspended in 10^{-6} solutions of the amines Valk and Price (1956) on the other hand found no catecholamines in human platelets It is difficult to escape the conclusion that these discrepancies are due to the choice of different methods by the three groups of workers The subject is obviously one which requires further work

ESTIMATION OF INDIVIDUAL CATECHOLAMINES IN BLOOD

Choice of Procedure

One of the difficulties of estimating catecholamines in peripheral plasma is that the concentrations are very near the threshold of sensitivity of all available methods Direct estimation of the amines by bioassay of native peripheral plasma of a resting subject is not possible it is however feasible in *adrenal vein blood* or in *perfusates* of organs during stimulation of adrenergic nerves Watts and Bragg (1957) found that during hemorrhagic shock levels of

epinephrine in peripheral plasma of dogs become high enough for direct determination Even in these circumstances assays of untreated plasma have the disadvantage that they will not give accurate values for the relative concentrations of epinephrine and norepinephrine though approximate figures can be obtained by so called parallel assays on two organs one of which is much more sensitive to epineph rine than to norepinephrine The ratio epinephrine to norepinephrine is then obtained by calculation but the error of the result may be high (Gaddum and Lembeck 1949) When bioassay of native plasma is attempted very strict precautions while collecting the blood are required since contamination with other pharmacologically active substances formed in shed blood particularly with 5 hydroxytrypta mine interferes with the assay The use of heparinized blood collected in cooled siliconed glassware and centrifuged as rapidly as possible at speeds which remove the platelets has given satisfactory results (Gaddum *et al* 1949) Even so the tissues used for the bioassay may have to be pretreated with antagonists of 5 hydroxytryptamine (see p 588)

Estimations of catecholamines in *peripheral blood* can normally only be carried out with the help of extraction procedures which concentrate the amines as purification proceeds The final extracts in which the amines are determined either fluorimetrically or bio logically may be prepared in such a way that they contain epinephrine and norepinephrine together or in separate fractions When epineph rine and norepinephrine are not separated the estimation of their relative quantity has to be done by some differential process followed by calculations which make use of two simultaneous equations One method employs oxidation at a different pH epinephrine being oxidized at pH 3 5 whereas norepinephrine requires a pH of 6 0 (Lund 1950 Euler and Floding 1955) the other makes use of dif ferences in the wavelengths of the emitted light of the fluorescent compounds formed by the two amines (Weil Malherbe and Bone 1954a Cohen and Goldenberg 1957a)

The alternative to the use of these differential procedures is the separation of epinephrine and norepinephrine before their estima tions here paper chromatography in phenol hydrochloric acid (James 1948) is the method of choice It is applicable to the minute quantities of epinephrine found in blood (Vogt 1952b Holzbauer and Vogt 1954) In practice the amines in the eluates from the paper have always been assayed biologically but there is no reason why fluori

metric estimations should not be applicable if the sensitivity of the method is stepped up sufficiently *It is obvious that separation of* the amines before estimation will increase the reliability of the results at the expense of prolonging the time required for the work It may be of interest to mention that the time necessary for obtaining ex tracting chromatographing eluting and assaying epinephrine in a small series of blood samples is two full working days

There are at present no generally accepted figures for blood con centrations of epinephrine and norepinephrine since the values depend both on the method used and on the experimenter Old values like those obtained by Kobro (1946) who used the reduction of arseno molybdic acid for his estimations are so high (44 μg per liter in human blood obtained with subject at rest) that they are now considered to be due to lack of specificity of the method The present choice lies between acceptance of the very low values given by bio-assay and some fluorimetric procedures and the higher figures ob tained by the majority of authors who use fluorimetric methods

Estimation Using Bioassay

Epinephrine The concentration of epinephrine in peripheral blood of conscious animals (and man) has been measured in two ways The most sensitive and also the most direct method was devised by Armin and Grant (1953 1955) The test consists in the observation of changes in caliber of the central artery of the denervated rabbit s ear The solution to be examined is injected directly into the distal end of the artery the effects can be calibrated by the injection of known amounts of epinephrine Epinephrine can be estimated in a concen tration of 10^{-11} and sometimes even 10^{-12} in a volume of 0 1 ml Freshly drawn rabbit blood or rabbit plasma can be assayed directly the blood may be drawn from any catheterized or exposed artery or vein of the donor into a heparinized nonwettable syringe and rein jected immediately into the ear of the test rabbit Arterial and peripheral venous *blood* contain an epinephrine equivalent of less than 0 01 μg per liter and blood from the inferior vena cava 0 1 to 1 0 μg per liter this activity disappears after adrenalectomy The adrenaline equivalent of peripheral plasma is just detectable (0 01 μg per liter) A brief struggle or a large hemorrhage raises the adrenaline 1 0 μg per liter The test does not allow differentiation between epi nephrine and norepinephrine in its present form it is not applicable to blood from other species

The second method has been used on rats rabbits dogs and man (Vogt 1952b Holzbauer and Vogt 1954 Muscholl and Vogt 1957) Blood is collected under the precautions mentioned on page 585 and plasma obtained by centrifugation at 3500 revolutions per minute In preparing an extract suitable for bioassay three conditions have to be observed (1) avoidance of oxidation of the catecholamines (for example by the use of acid solutions by the exclusion of contamination with heavy metals and by working at low temperatures) (2) preparation of a final extract free of any chemicals which have deleterious effects on the test organs such as acetates barium salts phenol etc and (3) concentration of the extract as much as is compatible with obtaining a final solution which is approximately isotonic with tissue fluids Extraction of the plasma with cooled acid ethanol has been found satisfactory this is followed by evaporation of the *solvent and purification of the residue which is then applied to acid* washed filter paper (Whatman No 1) for ascending chromatography in phenol hydrochloric acid Development of the chromatogram is carried out for 17 hours at 26 C the papers (from which the regions containing the markers should be cut off to avoid contamination of the whole paper with the markers) are washed in benzene to remove excess phenol This is followed by elution of the appropriate regions of the chromatogram with a sodium dihydrogen phosphate solution Details of the original procedures and of later modifications are found in Crawford and Outschoorn (1951) and Vogt (1952a) The technique of elution has since been further improved the elution time may be shortened to 2 hours by making the troughs holding the slides so shallow that the slides form only a small angle with the horizontal plane Complete elution requires collection of no more than 2 ml of eluate removal of the last traces of phenol is not done by shaking of the eluate with benzene but by making it up to 3 5 ml with washings of the collecting tube and evaporating it to complete dryness *in vacuo* at a bath temperature of 50 C

The assay of epinephrine is done on the rat s uterus by the method of Gaddum and Lembeck (1949) Certain modifications of the method may be required Sensitization of an insensitive preparation is achieved by treating the tissue for 10 minutes with a low concentration of dibenzyline (0 05 to 0 1 μg in a 2 ml bath) the sensitizing substance is then washed out but the effect persists (Holzbauer and Vogt 1955) In some species particularly in the rabbit the 5 hydroxytryptamine content of plasma is so high that in spite of careful blood collection

enough of this amine gets into the final extracts to interfere with the assays in the chromatogram 5 hydroxytryptamine travels only a little faster than epinephrine so that it contaminates the eluates of the epinephrine region This difficulty is overcome by antagonizing the action of 5 hydroxytryptamine with lysergic acid diethylamide 0 15 to 1 0 μg are left for 10 or 20 minutes in the 2 ml organ bath It is however necessary to assay the epinephrine against standards to which as much 5 hydroxytryptamine has been added as is present in the eluates (for details see Muscholl and Vogt 1957)

By these methods the epinephrine concentration in the plasma of rats was found to be between 1 0 and 6 0 μg per liter in conditions of severe stress no epinephrine was found in the plasma if the same stress was employed but the rats had been subjected to adrenal demedullation (Vogt 1952b) Conscious dogs injected with insulin showed concentrations of epinephrine ranging from 0 25 to 6 4 μg per liter of plasma at rest the epinephrine values lay between 0 06 and 0 25 μg per liter A single healthy human subject showed an epinephrine concentration of 1 8 μg per liter 45 minutes after an intravenous injection of insulin before the injection the amount of epinephrine had been <0 06 μg per liter (Holzbauer and Vogt 1954) Plasma epinephrine values in conscious rabbits varied between 0 05 and 4 0 μg per liter Since these animals were handled a great deal during the blood collections the high values are undoubtedly due to emotional disturbance the concentrations of epinephrine were greater still about one hour after an intravenous injection of reserpine (Muscholl and Vogt 1957)

Norepinephrine The presence of norepinephrine in peripheral plasma has not been demonstrated biologically but the bioassays have established an upper limit for its concentration The blood pressure of the pithed rat responds to 1 mμg of norepinephrine if the equivalent of 2 ml of plasma is injected into the rat and in spite of the fact that the extracts do not mask added norepinephrine no significant rise in pressure is observed the original concentration must lie below 0 5 μg per liter or if generous allowance is made for incomplete recoveries (Holzbauer and Vogt 1954) below 1 0 μg per liter Extracts of rabbit plasma occasionally produced small rises in blood pressure which corresponded to concentrations lying between 0 3 and 1 0 μg per liter With values so near the threshold of the method it was impossible to be certain whether these responses were indeed due to norepinephrine Rises in blood pressure which would have

corresponded to a plasma norepinephrine concentration of more than 1 0 μg per liter were not observed even under the experimental con ditions which led to the appreciable increases in plasma epinephrine described in the preceding paragraph The more sensitive method of Armin and Grant (1955) has not been employed after chromatographic *separation of the amines it should however be possible to detect* noradrenaline in peripheral plasma by that technique

Fluorimetric Estimations

Two methods have sufficient sensitivity to be applicable to estima tions of epinephrine and norepinephrine in blood the first is a method in which the fluorescent compounds adrenolutin and nor adrenolutin are formed from epinephrine and norepinephrine by adding an *oxidizing agent and then alkali Adrenolutin and nor* adrenolutin are trihydroxyindoles the method is therefore frequently called the THI *method and was devised by Lund (1950) and modi* fied by Euler and Floding (1955) Cohen and Goldenberg (1957a) have adapted it specifically for the estimation of epinephrine and norepi nephrine in blood these workers do not however as Lund had done differentiate between epinephrine and norepinephrine by oxidizing at different pH but by using the differences in excitation and emission spectra of the fluorescent lutines All details concerning procedures *may be found in their two papers* (Cohen and Goldenberg *1957a* b) Their results on peripheral (heparinized) venous blood of a series of normal human subjects are seen in Table I

TABLE I

Epinephrine and norepinephrine in venous plasma of normal individuals

	Epinephrine (μg /L)	*Norepinephrine* (μg /L)
Range	−0 05 − 0 19	0 09 − 0 50
Mean	0 06 ± 0 05	0 30 ± 0 07

The occurrence of occasional small negative values for epineph rine is due to the use of simultaneous equations and the slight errors inherent in the estimations

It is obvious that *Cohen and Goldenberg s* method gives figures for epinephrine which agree with the biological estimations the method is superior to the bioassay in that it permits the estimation of resting levels of norepinephrine the volumes of plasma required

are about 15 ml Valk and Price (1956) obtained essentially the same results (0 00 ± 0 01 μg per liter for epinephrine and 0 20 ± 0 10 μg per liter for norepinephrine) when they applied a similar method Price and Price (1957) amplified these findings by showing that the concentration of epinephrine found in venous plasma of man is not significantly greater than zero (0 01 ± 0 07 μg per liter) but that arterial plasma contains a detectable amount 0 10 ± 0 10 μg per liter The concentration of norepinephrine on the other hand is a little higher in venous than in arterial blood suggesting that some of the norepinephrine originates from the sympathetic nerves of the area drained by the vein The method is described in full detail in their paper It follows that according to these two groups of workers and in agreement with the bioassay epinephrine may or may not be found in peripheral blood depending on whether its concentration is above or below about 5×10^{-11} The concentration of norepinephrine appears to lie between 2 and 3×10^{-10}

In contrast to these results Weil Malherbe and Bone (1957) using the trihydroxyindole method obtained values which are about ten times higher This point will be discussed later

The second procedure by which catecholamines yield a strongly fluorescent compound is that discovered by Natelson *et al* (1949) of the condensation of the catecholamines with ethylenediamine (E D method) Since 1952 this E D method has been used by Weil Malherbe and Bone (1952a) for the determination of epinephrine and norepinephrine in blood The average values reported for venous blood in 6 fasting men were 2 5 μg per liter for epinephrine and 5 7 μg per liter for norepinephrine (Weil Malherbe and Bone 1954a) Not only were these figures surprisingly high but the method yielded the strange result that an injection of insulin which from all physiological evidence available *raises* blood epinephrine *lowered* it when the assessment was made by condensation with ethylenediamine (Weil Malherbe and Bone 1952b) Experiments carried out later using bioassay of epinephrine (Holzbauer and Vogt 1954) or the fluorescence of adrenolutin (Valk and Price 1956) gave the opposite result namely a rise in concentration of blood epinephrine after insulin These facts raised the suspicion that the ethylenediamine method may estimate other compounds in addition to epinephrine and norepinephrine and that it is their concentration which is lowered by an injection of insulin In a recent paper Weil Malherbe and Bone (1957) re examined and modified their method in an attempt at clarifying these contro-

versial points Before carrying out the fluorimetry they passed their extracts through columns which should have removed any catechol acetic acid or neutral catechols substances suspected to be present in blood and which would react with ethylenediamine They further searched for dihydroxyphenylalanine and for dihydroxyphenylethyl amine in blood without success They came to the conclusion that none of these compounds were present in amounts capable of causing an error in the estimation of epinephrine and norepinephrine They sup ported this conclusion by measurements carried out with the tri hydroxyindole method of Euler and Floding (1955) which in their hands and in contrast to the findings of Cohen and Goldenberg (1957a b) and of Valk and Price (1956) gave equally high figures as the use of the ethylenediamine method Though the new figures for epinephrine (1 1 ± 0 09 μg per liter) were lower than those reported in their previous paper the mean value for norepinephrine (1 1 ± 0 39 μg per liter) was little changed by the additional purification

Lastly the authors checked an aliquot of their final extracts pre pared for fluorimetry by bioassay on the rat s uterus The extracts of human plasma failed to show effects on the rat s uterus corresponding to the values for epinephrine found by fluorimetry the latter ranged from 40 to 240 mμg per milliliter of extract whereas by bioassay the two highest values were 5 and <10 mμg per milliliter Since no mask ing of the effect of epinephrine added to the extract was observed in the majority of instances the fact that bioassay gave very much smaller figures than the fluorescence remained unexplained The finding how ever that the final extract appeared to contain very little biologically active epinephrine is not in agreement with the suggestion made earlier (Weil Malherbe and Bone 1954b) that all the discrepancies between different methods could be accounted for by the amines carried in the platelets It is to be hoped that the authors who have done so much work in trying to test the specificity of their method will be able to discover the cause of this disconcerting lack of agree ment

With a view to avoiding cumbersome bioassays many authors have used Weil Malherbe and Bone s method or tried to modify it in such a way that it would give results more in keeping with the biological estimates All workers have reported considerably lower values than Weil Malherbe and Bone for epinephrine but the figures for nor epinephrine were of a similar order lying between 1 and 3 4 μg per milliliter depending on the author Manger *et al* (1954) Aronow

are about 15 ml Valk and Price (1956) obtained essentially the same results (0 00 ± 0 01 μg per liter for epinephrine and 0 20 ± 0 10 μg per liter for norepinephrine) when they applied a similar method Price and Price (1957) amplified these findings by showing that the concentration of epinephrine found in venous plasma of man is not significantly greater than zero (0 01 ± 0 07 μg per liter) but that arterial plasma contains a detectable amount 0 10 ± 0 10 μg per liter The concentration of norepinephrine on the other hand is a little higher in venous than in arterial blood suggesting that some of the norepinephrine originates from the sympathetic nerves of the area drained by the vein *The method is described in full detail in their* paper It follows that according to these two groups of workers and in agreement with the bioassay epinephrine may or may not be found *in peripheral blood* depending on whether *its concentration is above* or below about 5×10^{-11} The concentration of norepinephrine appears to lie between 2 and 3×10^{-10}

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1 22 μg per liter (range 0 37 to 2 30) Thus provided the $NaF/Na_2S_2O_3$ mixture is only added after the plasma has been separated off the ethylenediamine method gives the same results as the bioassay and the trihydroxyindole method

The substances which appear to be contributed by the cells may or may not be epinephrine and norepinephrine The results of the bio assays carried out by Weil Malherbe and Bone (1957) do not favor the view that they are Even if they were they would not represent amines in *plasma* It is the concentration in *plasma* which reflects the changing activities of the gland and which determines the effects of the secreted hormones on the peripheral organs As stated earlier equilibration between plasma and cells is a slow process and the values for whole blood and for plasma will only run parallel when the physiological conditions are static In Munro and Robinson s work subjects with complete transverse lesions of the spinal cord at or above the third thoracic segment were found to have very much lower figures for epinephrine and norepinephrine than normal subjects Assuming that the substances estimated were nothing but epinephrine and nor epinephrine one would expect that in this instance real plasma values and values obtained by adding $NaF/Na_2S_2O_3$ to whole blood would run parallel as in the absence of sympathetic innervation there should be no fluctuations in adrenomedullary secretion and therefore plenty of time for equilibrium between plasma and cells to be established

Burn and Field (1956) and Harley Mason and Laird (1958) have shown that the chemical basis of the reaction of catecholamines with ethylenediamine is very complicated and that more than one con densation product is formed this may explain why different modifica tions of the method give different results Condensation with ortho diamines of benzene and naphthalene instead of ethylenediamine has been suggested by Sulkowitch (1956) but there is at present no infor mation about the merits of this modification

One relieving feature in assessing usefulness of methods which ap- pear to lack specificity is that the physiological observations made with their help may agree with those obtained by other methods provided the changes in the levels of epinephrine and norepinephrine are greater than the changes which might occur in other substances giving the same chemical reactions Thus Richardson *et al* (1957) using the ethylenediamine method agree with Price (1957) who worked with

et al (1956) Millar (1956) Richardson *et al* (1956) Kagi *et al* (1957) and Gray and Young (1957) have described modifications of the ethylenediamine method and used them to examine plasma epinephrine and norepinephrine in normal human subjects the fall after reserpine (Burger 1956) and the rises occurring in cases of pheochromocytomas during anesthesia and after muscular work (Gray and Beetham 1957) With the original method of Weil Malherbe and Bone Griswold (1958) obtained resting concentrations for epinephrine and norepinephrine of 0 24 μg per liter and 3 2 μg per liter respectively these figures were raised by electroshock

In contrast to the preceding authors Valk and Price (1956) came to the conclusion that the ethylenediamine condensation method gives figures which may be correct for epinephrine but which are invariably about 10 times too high for norepinephrine They compared the two fluorimetric methods on the same samples of plasma and found norepinephrine concentrations of the order of 0 2 μg per liter by the trihydroxyindole (THI) method and figures which were about 10 times higher by the ethylenediamine method There is no explanation why Valk and Price found this discrepancy between the THI and the ethylenediamine methods whereas Weil Malherbe and Bone (1957) obtained the same results with the two procedures One difference which may be crucial is that Valk and Price added Weil Malherbe and Bone s anticoagulant solution of sodium fluoride and sodium thiosulfate to plasma whereas Weil Malherbe and Bone added the solution to the original blood Perhaps some compound not present in plasma but present in cells causes the trouble when the hypertonic $NaF/Na_2S_2O_3$ solution is in contact with cells This suspicion is confirmed by the results of Munro and Robinson (1958) and of Millar (1956) Munro and Robinson like Weil Malherbe and Bone collected whole blood in the $NaF/Na_2S_2O_3$ solution and then proceeded with an estimation by the trihydroxyindole method They obtained high values in normal human beings (epinephrine 2 3 μg per liter norepinephrine 4 2 μg per liter) which agree with those of Weil Malherbe and Bone

In contrast Millar (1956) using a modification of the ethylene diamine method added the $NaF/Na_2S_2O_3$ solution to the plasma He found that in resting normal subjects there was virtually no epinephrine in peripheral venous plasma He observed that the injection of insulin caused the plasma epinephrine to rise to a mean value of

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the trihydroxyindole technique in finding greater rises in blood nor epinephrine than in blood epinephrine during ether anesthesia That such agreement is however not always obtained is obvious from Weil Malherbe and Bone s finding of a fall in the levels of epinephrine in hypoglycemia

CONCLUSION

In animal experiments information about the activity of the adrenal medulla is most easily obtained by the older methods of examining the amine content of adrenal vein blood or the difference in hormonal content of a denervated and an innervated adrenal gland (Elliott 1912) this method makes use of the facts that most stimuli act only on the innervated adrenal medulla and that medullary hor mones are much more rapidly released than resynthesized Only when information on conscious animals is required or when the study applies to man does the estimation of catecholamines in peripheral blood become imperative The estimation of urinary excretion of cate cholamines useful in many studies of the physiology of the adrenal medulla and the sympathetic system does not reflect the extremely rapid changes in secretory activity of which the organism is capable It is for this type of work that estimations of epinephrine and nor epinephrine in peripheral blood are indispensable

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CHAPTER XVII

Gonadotropins of Extra-Pituitary Origin

Janet W McArthur

INTRODUCTION

HUMAN CHORIONIC GONADOTROPIN
- Chemical and biological properties
- Methods of assay
- Source
- Distribution and metabolism
- Excretion
- Levels in normal pregnancy
- Levels in abnormal conditions
  - Intrauterine fetal death
  - Ectopic pregnancy
  - Tumors of the chorion
  - Toxemias of pregnancy and diabetic pregnancy

PREGNANT MARES SERUM GONADOTROPIN
- Chemical and biological properties
- Methods of assay
- Source and distribution
- Metabolism and excretion
- Levels in pregnancy

BIBLIOGRAPHY

INTRODUCTION

In 1927 Aschheim and Zondek reported that the blood and urine of pregnant women contained a substance which when injected into immature female rodents induced the formation of ovarian blood points and corpora lutea within a period of 96 hours This gonad stimulating material initially called prolan was the first gonadotropin and one of the earliest of all hormones to be detected in body fluids

Widespread interest was aroused concerning the distribution of such gonadotropins among various classes of animals The body fluids of domestic and laboratory animals and of animals in zoological parks

HUMAN CHORIONIC GONADOTROPIN

Chemical and Biological Properties

The chemical and biological properties of HCG have been extensively investigated and reviewed (Li 1949 Evans and Simpson 1950 Cowie and Folley 1955 Morris 1955 Noble and Plunkett 1955) HCG is a glycoprotein and presumably a single substance which in addition to its amino acid constituents contains approximately 11 per cent of galactose and 5 per cent of hexosamine Although the hormone has been isolated from human urine in crystalline form the homogeneity of this and of other highly purified preparations has been questioned The properties of HCG as extracted from blood have received comparatively little attention (Fluhmann 1932a b 1933a b c 1936 Howard 1936 Boycott and Rowlands 1938 Noble *et al* 1939) However they appear to differ in no essential respect from those of urinary and placental extracts

In the species to which it is native chorionic gonadotropin exerts a luteotrophic action during early pregnancy (Hisaw 1944 Brown and Bradbury 1947) It prolongs the active life of the corpus luteum and enables it to continue the secretion of estrogen and progesterone until the placenta attains full competence in the elaboration of the sex steroids The possibility that other functions as yet undiscovered may be subserved by HCG is suggested by its persistence in the blood throughout gestation

Extraneous biological actions may be observed in human beings harboring hydatidiform moles and malignant tumors which contain actively proliferating chorionic tissue Substances similar to if not identical with HCG gain access to the circulation of such patients and may like HCG injected into the nonpregnant human being exert a gonadotropic effect in conjunction with endogenous pituitary gonadotropic hormones Under these circumstances the corpus luteum interstitial tissue and follicular apparatus of the female and the Leydig cells and seminiferous tubules of the male gonad may all respond to some extent

In subprimate species to which chorionic gonadotropin is a foreign substance the hormone evokes a multiplicity of gonadotropic effects Some of these are dependent upon the simultaneous presence of endogenous pituitary gonadotropin while other reactions are elicited independently Advantage is taken of these biological responses to

were promptly subjected to Aschheim and Zondek s test for pregnancy Both the blood and urine of the gravid sheep goat pig cow dog cat *mouse rat rabbit and* guinea pig yielded disappointingly negative results (Zondek and Aschheim 1927 Zondek 1929 1931 Ehrhardt and Ruhl 1933 Asdell and Madsen 1933 Kust 1934) The blood and urine of the elephant (stage of pregnancy not stated) were found by Zondek (1929) to be devoid of gonadotropin as were the urines of pregnant lions tigers and ferrets (Ehrhardt and Ruhl 1933) However the blood and urine of the rhesus monkey and orangutan (Ehrhardt and Ruhl 1933 Zondek 1935 Delfs 1941) and of certain Equidae (see below) and the urine of the chimpanzee (Zuckerman 1935) proved to contain demonstrable gonadotropic activity during pregnancy An extensive bibliography of this widely scattered literature is provided by Cowie (1948)

Prolan was originally thought to be derived from the anterior lobe of the pituitary gland However it soon became apparent that the biological properties of this substance more closely resembled those of placental than of pituitary extracts Evidence pointing to the fetal chorion as the source of primate gonadotropin and the maternal endometrium as the source of pregnant mare s serum gonadotropin is now convincing Accordingly the term human chorionic gonadotropin (HCG) has supplanted both the original name and the intermediate name of anterior pituitary like hormone

Despite the negative results of direct assay indirect evidence is accumulating which suggests that the circulation of other animal classes besides the Primates and Equidae may contain gonad stimulating materials during pregnancy (Astwood and Greep 1939) The placenta of the rat mouse and hamster contains luteotrophic substances and evidence of a somewhat more circumstantial character (see under Pregnant Mare s Serum Gonadotropin page 613) tends to implicate additional classes of animals It is conceivable that with the aid of more satisfactory assay methods the majority of eutherian mammals will eventually be shown to elaborate distinctive pregnancy gonadotropins

Because of the important differences which obtain between primate and equine pregnancy gonadotropins these substances will be discussed separately

was undertaken and yielded results expressed in animal units Despite its limitations this approach permitted recognition of the large differences in blood HCG levels which obtain during the different trimesters of normal pregnancy and in various pathological states

In 1938 an international standard for HCG prepared from pregnancy urine was adopted It was emphasized that the reference material the gonadotropic activity of 0 1 mg of which was defined as the international unit should be employed for recording the activities of all preparations of human pregnancy urine *but only of such* However the manifest advantages of a standard preparation soon led to its extension to the assay of plasma serum and extracts of blood It was assumed that the reference standard and the material to be assayed constituted dilutions of HCG in different but biologically inert diluents

Evidence which created doubt concerning the propriety of such an assumption was shortly obtained by Rubin *et al* (1946) These investigators who employed rat uterine weight as the index of response to HCG noticed a significant difference in the slope of the dose response curves for pregnancy serum and the international standard In assays of serum HCG by the 4 hour ovarian hyperemia method Albert (1948) and Albert and Berkson (1951) observed an inhibitory protein effect which could be overcome by making a 1 10 dilution of the serum before injection The converse effect was noted by Maddock and Leach (1952) in the course of studies concerning the effects of HCG upon the human testis They found that 24 hours after a single intramuscular injection the hormonal activity accounted for in the plasma appeared to exceed the total administered dose In subsequent studies (Maddock *et al* 1953 Leach *et al* 1954) they reported a five to tenfold increase in the activity as determined by the rat uterine weight method if the hormone was administered together with plasma serum egg white or polyvinyl pyrrolidone The effect did not occur when the augmenting substances were administered at a site separate from the hormone

European investigators have refined and extended these observations (Table I) Diczfalusy and Loraine (1955) have noted a twofold increase in the activity of HCG dissolved in serum as against saline when assayed by the uterine weight method No such potentiation was observed in assays employing the weights of the male accessory sexual organs Since both assays extended over periods of several days delayed adsorption or inactivation might logically have been expected

detect and titrate the hormone Unfortunately none of the gonadotropic effects so far observed in assay animals can be regarded as absolutely specific for HCG similar reactions can in varying degrees be elicited by gonadotropins of pituitary and endometrial origin

Methods of Assay

The methods which have been developed for the assay of HCG have been extensively reviewed (Thayer 1946 Hamburger 1950 Loraine 1952 1956 1958 Diczfalusy 1953 1954) Diczfalusy and Heinrichs (1956) have appraised the sensitivity of several methods in current use and Borth and de Watteville (1952) have collected data illustrating the influence of animal strain experimental technique and time of reading upon sensitivity The accuracy of the various methods employed in defining the international unit of HCG was calculated by Emmens (1939a) Loraine (1950a) and Diczfalusy and Heinrichs (1956) have subsequently determined the precision of a number of additional methods The inadequacy of many of the techniques employed by earlier investigators particularly in the clinical field has been pointed out by Loraine (1953) Reference should be made to these papers in evaluating the significance of the data summarized in this chapter

For the detection and quantitation of HCG blood is in certain respects superior to urine In forensic medicine fragments of cloth containing dried blood stains which cannot be satisfactorily extracted by elution may be implanted subcutaneously in test animals (Goroncy 1932) In clinical medicine blood samples permit positive identification of the donor The pioneer studies which indicated that HCG is readily detectable in the blood (Fels 1927 Zondek 1931 Fluhmann 1929 1930) have been amply confirmed Serum has been shown to yield results which are at least as reliable as those obtained with urine (Brown 1932 Hofmann 1932 di Cioia 1937 Sutherland and Zwarenstein 1939 Evans and Krajian 1944 Rosenfeld and Rosenfeld 1944 Smith and Smith 1944 Foote and Jones 1946 Allison 1954 Benitez *et al* 1954) In addition because of its lesser toxicity serum more frequently permits the re use of animals

In the years which followed the discovery of HCG the inadequacy of purely qualitative determinations particularly in differentiating hydatidiform mole and choriocarcinoma from normal pregnancy was soon apparent Rough quantitation by the method of serial dilutions

Source

The trophoblastic shell of the chorionic villus is generally regarded as the site of origin of HCG However it has not been finally determined whether the hormone is synthesized by the cytotrophoblast the syncytial trophoblast or both The parallelism between the level of circulating HCG and the rise and decline of the cytotrophoblast (Wislocki and Bennett 1943 Wislocki *et al* 1948 Baker *et al* 1944) and the elaboration of HCG by tissue cultures in which only the cells of the cytotrophoblast survive (Jones *et al* 1943) tend to favor this tissue as the source of the hormone The histochemical techniques which have so far been applied to placental tissue lack the specificity required to furnish decisive evidence on this point

The sudden rise in the concentration of circulating HCG which takes place during the first trimester of pregnancy is attributed by Newton (1938) to the establishment of the circulation in the chorionic villi However in Wislocki s opinion (1943) this places the establishment of the circulation rather late In the rhesus monkey the circulation becomes established soon after the twenty second day (Wislocki and Streeter 1938) towards the close of the brief period during which gonadotropic hormone can be detected in the blood (Delfs 1941) The high concentration of HCG in placental tissue during the period of pregnancy when maximal levels of HCG are detectable in the serum (Diczfalusy 1953) also tends to refute Newton s contention

The quantitative relation between placental and serum concentrations of HCG is such as to indicate that the hormone is continuously released into the blood stream (Diczfalusy 1953) No appreciable diurnal variation in the level of circulating HCG is demonstrable (Borth *et al* 1958)

Distribution and Metabolism

Data concerning the distribution of HCG are not extensive However the available information (Parker and Tenney 1940 Bruner 1951) indicates that the maternal blood ranks next to the fetal chorion with respect to HCG concentration HCG is detectable in maternal muscle (Bruner 1951) and presumably permeates all tissues Although a number of investigators (Geist and Spielman 1934 Soule 1934 Bourg and Legrand 1935 Parker and Tenney 1940) were unable to detect the hormone in the fetal circulation others (Fels 1927 Bruhl

TABLE I Potency of HCG Dissolved in Serum or Plasma Compared with That Dissolved in Saline as Estimated by Various Methods of Assay †

M thod	C ntrat f rum % (/)	M an p tency at fidu i l limits % (p 0 05)			R f enc
(a) Tests in which m an pot cy ratio of 100% s with n the fid cial ng					
Total prostatic we ght (intact ats)	100	106	84	132	
S minal vesicle weight (i act rats)	100	114	80	199	D c fal sy a d Lo a ne (1955)
Vent l p static weight (hypophys ctomized rats)	100	115	75	192	
(b) Te ts in whi h a me n p tency t of 100% i o ts de of th f d ci l a ge					
Uteri e weight (intact rat)	100	188	157	225	Diczfal sy nd Lo a ne (1955)
Spe miation (Bufo viridi)	100	170	150	190	Lun feld et l (1957)
Spe miation (Rana e c lenta)	50 100	30 20	10 1	60 30	Salvati r a a d To res (1952)
(c) T ts in whi h the f duci l ng is d p de t upon th nce tr t or of rum jected					
Ovari hype mia (4 h r te t)	10 25 100	100 De as g to 30% a erum on cent t o i c ased	—	— —	Alb rt nd Berkso (1951)
Ovar a hype mia (16 ho t t)	35 70 100	90 100 70	60 90 50	100 110 90	Borth et al (1957)

Th poten y f HCG i 0 9 p ent NaCl i as um d to b 100 p ent
† Afte Borth t al (1957) nd Lo aine (1957)

to augment the effect in both sexes Hamburger (1957) has suggested that biological synergism between the HCG and small amounts of pituitary follicle stimulating hormone contained in the serum may explain the discrepancy

Whatever the precise mechanisms involved it is clear that the en hancing or inhibiting effect of serum introduces a distinct bias in certain methods employed for the assay of HCG Preference should be given to methods which are free from this bias or alternatively the bias should be eliminated by administering both the standard and unknown preparations in the same vehicle

Source

The trophoblastic shell of the chorionic villus is generally regarded as the site of origin of HCG However it has not been finally deter mined whether the hormone is synthesized by the cytotrophoblast the syncytial trophoblast or both The parallelism between the level of circulating HCG and the rise and decline of the cytotrophoblast (Wis locki and Bennett 1943 Wislocki *et al* 1948 Baker *et al* 1944) and the elaboration of HCG by tissue cultures in which only the cells of the cytotrophoblast survive (Jones *et al* 1943) tend to favor this tissue as the source of the hormone The histochemical techniques which have so far been applied to placental tissue lack the specificity re quired to furnish decisive evidence on this point

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1929 Zondek 1931) have *found it to be present in low concentration* Sklow (1942) obtained an average ratio of 23 1 between the HCG content of retroplacental and umbilical vein blood whereas Bruner (1951) observed that the ratio of concentration between maternal muscle and fetal trunk tissue varied between 10 1 and 20 1

Electrophoretic studies of the serum of pregnant women suggest that HCG is transported in association with the β globulins (Demol and Fanard 1957)

The metabolic fate of circulating HCG is largely unknown The rate of inactivation of HCG injected into animals is expressed by a logarithmic curve The scattered reports which Zondek and Sulman (1945a) have collected indicate that the greater part of the injected *hormone is rapidly destroyed possibly by the reticulo endothelial sys* tem and that only 5 to 10 per cent appears in the urine The inactiva tion of HCG administered to nonpregnant human beings appears to occur at a slower rate However the data available on this point are meager (Friedman and Weinstein 1937 Bradbury and Brown 1948 Lloyd *et al* *1949* Pedersen Bjergaard and Tønnesen *1950* Leach *et al* 1954)

The disposal of HCG in the human female during the immediate post partum period has been studied by Wilson *et al* (1949) John son *et al* (1950) and Hinglais and Hinglais (1953) The Mayo group found the serum to be cleared of the hormone within a period of 9 hours However only 5 8 per cent of the total quantity of HCG cal culated to be present in the circulation at the moment of delivery of the placenta could be accounted for in the urine Salvatierra (1954) recovered 7 7 per cent of the estimated blood HCG in the urine col lected post partum Bruner (1951) and Hinglais and Hinglais (1953) have followed the disappearance of circulating maternal HCG follow ing therapeutic abortion

Excretion

The urinary excretion of HCG by patients transfused with pregnant women s blood begins within 10 minutes of the start of the infusion and continues for 12 to 24 hours During the first 2 to 20 hours sufficient quantities of HCG are present in the circulation to cause luteinization of the ovaries of test animals Amounts adequate to pro duce follicle stimulation only are present for a considerably longer period (Ehrhardt 1930)

The coefficient of elimination of HCG was determined by Bourg

and Legrand (1935) in a pioneer study which may mark the first attempt ever made to quantitate the renal clearance of a hormone Clearance values derived from their data by the formula UV/B are in excellent agreement with those obtained by later investigators

Clearance studies have established the fact that the fluctuation in the HCG concentration of body fluids during normal pregnancy depends upon changes in the rate of secretion or inactivation of the hormone rather than upon changes in the rate of urinary excretion (Table II) Although the absolute values may reflect the methodological difficulties mentioned earlier the comparative constancy of the results obtained during the different trimesters with any given method is evident

TABLE II Renal Clearances and Serum to Urine Ratios of HCG Obtained During the Various Trimesters of Pregnancy in Normal Women

Method	Clear ml. p m T imest rs			Serum to ur ti T imest			Ref nc
	I	II	III	I	II	III	
Ovarian hype mia (4 ho r t t)	0 41	0 38	0 36	2 93	3 24	2 62	G t et al (1949)
Total pr stati w ight (tact rat)	0 91	0 93	0 98	0 83	1 16	1 21	Lo e (1950b)
Sperml tio (Rana escul ta)	0 59	0 51	0 48	1 46	1 86	1 81	Sal ti a (1954)

Levels in Normal Pregnancy

Attempts to achieve an accurate titration of circulating HCG during the course of gestation began soon after the discovery of the hormone and continue to date An early impression that the concentration of HCG rises steadily throughout the course of pregnancy (Kennedy 1933) was soon corrected Smith and Smith (1935a) described the occurrence of peak levels during the second third and fourth months and a subsequent drop to levels which remained fairly constant throughout the remainder of pregnancy In a study which dealt mainly with urinary excretion but which included a few serum levels Evans *et al* (1937) emphasized the sharpness of a peak which they detected between 50 and 60 days after the last menstrual period The emerging pattern of a first trimester peak and a subsequent plateau was confirmed by Boycott and Rowlands (1938) Rakoff (1939a b) and Fosco (1943) Trettenero (1936) and Behrman and Niemann (1955) described a lesser peak toward the middle of the last trimester

Jones *et al* (1944) were the first to express the results of serial determinations in terms of the international standard rather than in animal units They employed the rat uterine weight method to assay extracted serum Subsequently Albert (1948) Albert and Berkson (1951) and Behrman and Niemann (1955) have made quantitative determinations on unextracted serum with the aid of the rat ovarian hyperemia test Haskins and Sherman (1952) with the spermiation reaction in *Rana pipiens* and Loraine (1957) with the rat prostatic weight method (Fig 1) The resulting composite curves are in good agreement with

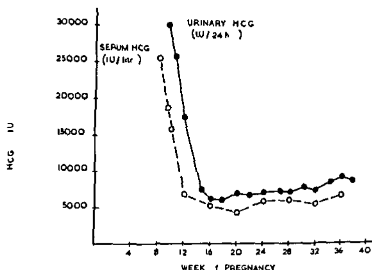


FIGURE 1 Mean estimations of urinary and serum HCG throughout normal pregnancy The curve for urinary excretion was constructed on the basis of 136 determinations Four patients were followed throughout pregnancy at approximately 2 week intervals and scattered determinations were made in a further 10 patients (Loraine 1950a) The curve for serum concentration was constructed on the basis of single determinations in 107 subjects (Loraine personal communication) The total prostatic weight of the immature rat was employed as the response criterion (Reproduced from Loraine J A *The Clinical Application of Hormone Assay* E and S Livingstone Ltd Edinburgh and London 1958)

respect to their general configuration However the absolute levels obtained exhibit considerable variation This is not surprising in view of the differences in precision and in susceptibility to serum bias of the various assay techniques which were employed

Changes in serum HCG concentrations during early pregnancy and during the last trimester have been the subject of special study The time of first appearance of HCG has been determined by Smith *et al* (1951) (Table III) The frequency with which the hormone becomes

TABLE III Earliest Day of Positive Test for HCG During 15 Pregnancies in 11 Women

Day†	Number of positive tests Serum	Urine
10	0	0
9	1	1
8	1	0
7	1	0
6	1	1
5	6	6
4	3	4
3	0	1
2	2	1
1	0	0
0	0	1

After Smith et al (1951)

†Days prior to the expected but missed menstrual period

detectable five days prior to the expected recurrence of menstruation accords well with the available anatomical data The human blastocyst begins erosion of the maternal endometrium on the sixth day and the trophoblast has been detected entering a maternal sinusoid on the ninth day after ovulation (Rock and Hertig 1948) Little change occurs in serum HCG levels between the twenty fourth and fortieth days after the last normal menstrual period (Smith *et al* 1951) After day 40 there is a progressive rise in titer which reaches a maximum on approximately day 70 by day 90 the titer has fallen to a comparatively steady state

During the later stages of pregnancy Pedersen Bjergaard and Pedersen Bjergaard (1948) observed constant or slightly rising titers of serum HCG while others (Taylor and Scadron 1939 Stroink and Muhlbock 1948) noted variable levels Albert and Berkson (1951) have compared their own data for this period with those of Jones *et al* (1944) and of Smith and Smith (1934 1935a 1937 1941a b) The levels noted by the Smiths showed a slight tendency to rise and those of Jones *et al* to fall terminally Albert s own data exhibit little change The resultant is a somewhat variable level with no consistent trend in either direction

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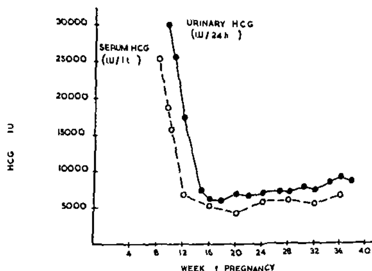


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ing moles which are in close contact with maternal structures exhibit a high titer of serum HCG (Payne 1941 Zondek 1942 Frank 1943 Haskins and Sherman 1952 Behrman and Niemann 1955) The levels attained are greatly in excess of those characteristic of normal preg nancy except at the physiological peak Moles which are undergoing degeneration (Gastineau *et al* 1949) or which are isolated from the maternal circulation by a layer of fibrin release appreciably lesser amounts of HCG Indeed cases in which the urine gave a negative Aschheim Zondek test have been reported

Choriocarcinoma (chorionepithelioma) a malignant tumor of the chorion is a rare condition which most commonly follows a pregnancy and involves the uterus Fifty per cent of such cases follow hydatidi form mole 25 per cent follow abortion and 25 per cent develop after a full term pregnancy When choriocarcinoma occurs in the absence of gestation it is believed to arise from the trophoblastic elements of an ectopic teratoid embryo Such tumors most commonly originate along the path of germ-cell migration or the distribution of the em bryonic genital ridge (i e in the ovary testis retroperitoneal space or mediastinum)

In patients with choriocarcinoma of the uterus the serum level of HCG is frequently but not invariably elevated to great heights (Smith and Smith 1935b Zondek 1937 Payne 1941) Serial studies delineat ing the fall of serum HCG after the expulsion of nonmalignant moles the return of the hormone associated with the development of chorio carcinoma and the final disappearance of HCG from the circulation of patients cured by hysterectomy have been made by Brindeau *et al* (1935) and by Delfs (1957) The latter provides criteria for the diag nosis of hydatidiform mole and for that of chorionepithelioma follow ing such a mole based on the long term investigation of a large number of patients All serum gonadotropin titers are expressed in international units and the superiority of quantitative techniques is brilliantly demonstrated

A pronounced drop in the serum HCG level was observed by Smith and Smith (1935b) in a dying patient to whom they administered theelin (estrone) In a patient with metastatic disease treated with x ray and hexestrol by Hinglais and Hinglais (1948) a precipitous fall in the level of serum HCG occurred just before death Neither testosterone alone nor in combination with stilbestrol prevented a terminal rise in the level of serum HCG in a similar patient studied by Haskins and Sherman (1952)

Multiple pregnancies may be accompanied by elevated serum HCG levels Jones *et al* (1941) obtained high values throughout one mono zygotic twin pregnancy and moderately high levels during the first half of gestation only in a dizygotic twin pregnancy The total weight of the placental tissue delivered by the two patients was almost *identical* Corroborative data have been reported by Stroink and Muhlbock (1948) Haskins and Sherman (1952) and Behrman and Niemann (1955)

Levels in Abnormal Conditions

Intrauterine Fetal Death A progressive fall in serum HCG levels accompanies intrauterine fetal death (Fluhmann 1930 Brindeau *et al*, 1933 Chosson and Donnet 1931 Bourg and Legrand 1935 Rakoff 1940) During early pregnancy the imminence of this complication can sometimes be recognized by a failure of the serum HCG levels to exhibit the rise which normally occurs between days 10 and 69 (Smith *et al* 1951) In habitual rather than incidental abortion serial studies of serum HCG levels reveal several different patterns (Dells and Jones 1948) Normal levels of HCG do not preclude abortion However con sistently low levels are incompatible with a successful pregnancy

Serial HCG levels in patients who carry pregnancies *to term follow* ing a previous abortion tend to fall earlier and more precipitously and urinary pregnanediol levels to rise slightly later than in patients who have experienced no previous reproductive difficulties (Jones and Dells 1951)

Ectopic Pregnancy Serum HCG levels below those considered normal for intrauterine pregnancy of the same age are frequently (Fluhmann 1930 Chosson and Donnet 1931 Ramos and Collazo 1941) but not invariably (Haskins and Sherman 1952) observed in patients with ectopic pregnancy Since abortion is generally in progress at the time of study it is difficult to determine the extent to which the lower HCG levels reflect deficient trophoblastic activity at the implantation site

Blood obtained from the peritoneal cavity of patients with ectopic pregnancy was found by Ezes and Bourgarel (1952) to contain demon strable HCG although tests on samples of circulating blood obtained concomitantly were negative

Tumors of the Chorion Hydatidiform mole a benign tumor of the chorion is a comparatively rare condition which is believed to result from a defect in the fetal vascular anlagen Patients with actively grow

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Toxemias of Pregnancy and Diabetic Pregnancy The toxemias of late pregnancy may be accompanied (Smith and Smith 1933) and may sometimes be preceded (Smith and Smith 1934 1941a Rakoff 1939b) by moderate elevations in the level of circulating HCG Extensive studies by Smith and Smith (1935a 1939 1941a 1947 1948) and others (Taylor and Scadron 1939 Rakoff 1939b Loraine and Matthew 1950) have shown that increased levels of HCG are more common in patients with eclampsia and severe pre eclamptic toxemia than in the milder cases Concomitant increases in placental HCG have been noted (Smith and Smith 1935c Loraine and Matthew 1953) Accompanying decreases in renal HCG clearance have been observed by Loraine (1950b) whereas Lajos and Pali (1951) noted increases in HCG clearance There is little apparent correlation between increased HCG levels and specific clinical features such as hypertension convulsions edema or albuminuria (Smith and Smith 1934 1935a Rakoff 1939b Taylor and Scadron 1939 Loraine and Matthew 1950) The administration of estrogen alone or in combi nation with progesterone has been followed in some instances (Smith and Smith 1938 1940 1941b Siegler 1939) by a fall in serum HCG

Moderate increases in the level of serum HCG are likewise observed in a proportion of diabetic pregnancies (Smith and Smith 1937 1938 1939 1940 1941a b) The incidence has been reported to be high by some (White *et al* 1939 White and Hunt 1943 White 1952) and low by other investigators (Rubin *et al* 1946 Loraine and Matthew 1954) As in the case of toxemias in nondiabetic patients the absence of agreement appears at least partially attributable to differences in the precision and serum bias of the assay techniques (Loraine 1953 1956 1957) and in the extent to which statistical methods were em ployed in the analysis of the results (Cohen *et al* 1943)

The finding that fetal death pre eclamptic toxemia and premature delivery occur almost exclusively in pregnant diabetics with elevated rather than normal HCG levels (White *et al* 1939 White and Hunt 1943 White 1952 Jordan 1943) has not been confirmed by other

investigators (Smith *et al* 1944 Keltz *et al* 1950 Loraine and Matthew 1954) A decrease in serum HCG levels during treatment with estrogen and progesterone was obtained by Smith and Smith (1938 1940) and by White and Hunt (1943) but not by Keltz *et al* (1950) Loraine (1949) noted an appreciable but transient fall in urinary HCG excretion during the course of estrogen therapy Placental concentrations of HCG tend to be increased (Loraine and Matthew 1953) and renal clearances to be decreased (Loraine 1950b) in diabetic pregnancy

The studies of McKay *et al* (1958) may explain the frequent rise of HCG in diabetic and toxemic pregnancy and also the occasional rise of HCG during the later stages of normal pregnancy These investigators have noted a reappearance of the Langhans cells in the fibrin trapped *anoxic villi of the aging placenta*

PREGNANT MARE S SERUM GONADOTROPIN

The presence of a gonad stimulating substance in the serum of the mare during certain stages of pregnancy was discovered independently by Cole and Hart (1930a) and by Zondek (1930) The name equine gonadotropin proposed by Cole *et al* (1940) is less frequently em ployed than is the cumbersome but more precise term pregnant mare s serum gonadotropin and its abbreviation PMS Pregnancy gonadotropins with similar properties have been detected in the serum of the donkey (Zondek and Sulman 1945b) zebra (Zondek 1935) and fallow deer (Unterberger 1932) and in the urine of the giraffe (Wilkinson and de Fremery 1940)

Chemical and Biological Properties

The chemical (Goss and Cole 1931 Li 1949 Morris 1955) and biological (Cole *et al* 1932 Cole 1936 Engle 1939 Evans and Simp son 1950 Cowie and Folley 1955 Noble and Plunkett 1955) proper ties of PMS have been the subject of extensive study It is a remarkable fact that no biological differences have so far been detected between the gonadotropic activity of pregnant and nonpregnant mare s serum (Cole and Goss 1939 Fraenkel Conrat *et al* 1940 Cole 1946) Although a homogeneous preparation has yet to be isolated the similarity of the gonadotropic effects elicited by fractions prepared from PMS and by PMS itself suggests that they are mediated by a single entity (Cole *et al* 1940) The hormone is a glycoprotein which

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Toxemias of Pregnancy and Diabetic Pregnancy The toxemias of late pregnancy may be accompanied (Smith and Smith 1933) and may sometimes be preceded (Smith and Smith 1934 1941a Rakoff 1939b) by moderate elevations in the level of circulating HCG Extensive studies by Smith and Smith (1935a 1939 1941a 1947 1948) and others (Taylor and Scadron 1939 Rakoff 1939b Loraine and Matthew 1950) have shown that increased levels of HCG are more common in patients with eclampsia and severe pre eclamptic toxemia than in the milder cases Concomitant increases in placental HCG have been noted (*Smith and Smith 1935c Loraine* and Matthew 1953) Accompanying decreases in renal HCG clearance have been observed by Loraine (1950b) whereas Lajos and Pali (1951) noted increases in HCG clearance There is little apparent correlation between increased HCG levels and specific clinical features such as hypertension convulsions edema or albuminuria (Smith and Smith 1934 1935a Rakoff 1939b Taylor and Scadron 1939 Loraine and Matthew 1950) The administration of estrogen alone or in combination with progesterone has been followed in some instances (Smith and Smith 1938 1940 1941b Siegler 1939) by a fall in serum HCG

Moderate increases in the level of serum HCG are likewise observed in a proportion of diabetic pregnancies (Smith and Smith 1937 1938 1939 1940 1941a b) The incidence has been reported to be high by some (White *et al* 1939 White and Hunt 1943 White 1952) and low by other investigators (Rubin *et al* 1946 Loraine and Matthew 1954) As in the case of toxemias in nondiabetic patients the absence of agreement appears at least partially attributable to differences in the precision and serum bias of the assay techniques (Loraine 1953 1956 1957) and in the extent to which statistical methods were employed in the analysis of the results (Cohen *et al* 1943)

The finding that fetal death pre eclamptic toxemia and premature delivery occur almost exclusively in pregnant diabetics with elevated rather than normal HCG levels (White *et al* 1939 White and Hunt, 1943 White 1952 Jordan 1943) has not been confirmed by other

day it has disappeared completely from the circulation This evidence though circumstantial implies that a function of PMS may be to stimulate the formation of accessory corpora lutea which maintain the endometrium in a state favorable for implantation during the period when the primary corpus luteum is on the wane (Cole *et al* 1931 Kimura and Lyons 1937 Amoroso 1955)

The presence of accessory corpora lutea in the ovaries of the pregnant nilgai (Amoroso 1955) and African elephant (Perry 1953) suggests that in these species certain stages of gestation may be sustained by a similar mechanism

In nonequine species to which PMS is a foreign substance the hormone is capable of eliciting the full complement of gonadotropic effects whether or not endogenous pituitary gonadotropins are present In the female organism follicle growth ovulation and corpus luteum formation occur and in the male spermatogenesis and interstitial cell function are stimulated The biological versatility of PMS as compared with HCG is consonant with its more diverse functions in its native species

Methods of Assay

None of the methods which have so far been developed for the assay of PMS can be regarded as absolutely specific The characteristics of these methods have been reviewed by Thayer (1946) and by Hamburger (1950)

In 1939 the international unit for PMS was defined as the gonadotropic activity of 0 25 mg of the reference standard The accuracy of the various methods employed for the definition of the international unit was determined by Emmens (1939b) He found that tests which employed ovarian weight as the response criterion were superior to those utilizing other criteria Additional data concerning the precision of the ovarian weight method have been provided by Jacobsohn (1949)

The absence of practical difficulties in assaying commercial PMS preparations of varying degrees of purity against the standard preparation argues in favor of the qualitative constancy of the hormone (Hamburger 1950)

Source and Distribution

Equine gonadotropin was at first thought to be secreted by the pituitary gland or like HCG by the fetal chorion (Catchpole and

resembles HCG in that it contains galactose and possesses a hexose/hexosamine ratio of approximately 2 1

Clarification of the physiological role of PMS requires a short digression concerning pregnancy in equines Implantation of the blastocyst occurs on or about the fortieth day of gestation which in the mare has a duration of 329 to 345 days (Eckstein and Zuckerman 1956) The period of activity of the equine corpus luteum unlike that of most other mammals is brief and does not greatly exceed that of the estrous cycle of the nonpregnant animal toward the end of the first month of gestation the corpus luteum begins to regress (Amoroso 1955) However during the second and third months a fresh set of corpora lutea make their appearance following supplementary ovulations (Amoroso *et al* 1948) and perhaps in part as a result of luteal atresia of developing follicles (Kimura and Lyons 1937) As many as eleven fresh corpora lutea have been found in the ovaries (Clegg *et al* 1954) By the one hundred twentieth day follicular growth ceases although the corpora lutea persist for another month or more Thereafter the maternal ovaries become so quiescent as to appear fibrotic (Eckstein and Zuckerman 1956)

The time relationships which obtain between PMS concentration in the serum and ovarian activity are depicted in Figure 2 It will be noted that the hormone is not detectable until approximately the fortieth day of gestation that it reaches peak levels during the second and third months of pregnancy and that by the one hundred eightieth

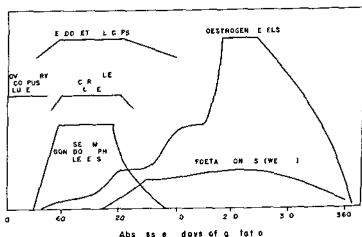


FIGURE 2 The events during gestation in the mare (Reproduced from Asdell S A *Patterns of Mammalian Reproduction* 1946 by courtesy of the publishers Comstock Publishing Co Inc Ithaca New York)

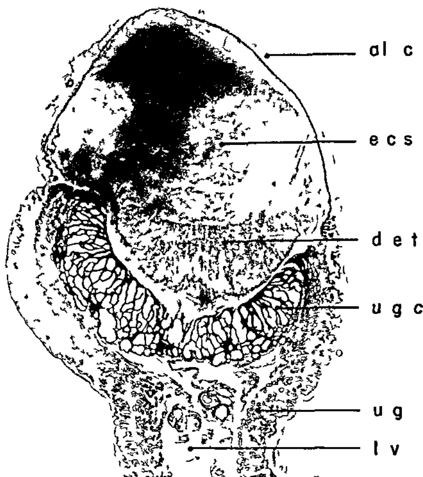


FIGURE 3 Endometrial cup from mare No 4 sacrificed on the 105th day of gestation At this stage of pregnancy the cups are pendulous Note the enlargement of the lumina of the uterine glands in the cup area al.c allantochorion e c.s endometrial cup secretion d e t degenerated endometrial tissue u g c uterine glands in cup area u g uterine glands outside of cup area l v lymphatic vessel Professor E C Amoroso who has studied the lymphatic transport of equine pregnancy gonadotropin (see text) kindly identified the lymphatic vessel in this illustration At higher magnification the lumen of the vessel is seen to contain a jelly like coagulum the secretory product of the endometrial cup (Reproduced from Cole H H and Goss H The source of equine gonadotrophin In *Essays in Biology in honor of H M Evans* University of California Press 1943)

Lyons 1933 1934) However the evidence (Cole and Goss 1943 Clegg *et al* 1954 Amoroso 1955) points increasingly to specialized *endometrial structures known as* endometrial cups as the source of the hormone The cups are limited to the fertile horn and occupy a circular area of the endometrium in the neighborhood of the yolk sac They contain a viscous gel which forms a protuberance covered by the chorion (Fig 3)

The view that the cups constitute the site of formation of PMS is supported by the following observations (1) The appearance of the hormone in the blood *coincides with the development of the cups* and its disappearance with their desquamation (2) in the early stages of their development the cups contain the hormone in a concentration higher than that of the blood and (3) the uterine mucosa contains gonadotropin only in the cup area

The prominence of the lymphatic tissue and lymphatic vessels in the base of the endometrial cups suggested to Amoroso (1959) that the gonadotropin might gain access to the maternal circulation via lymph channels He cannulated the thoracic duct and found the gonado tropin concentration of lymph to be many times that of the serum of the pregnant mare

In the opinion of Clegg et al (1954) the endometrial cups act mainly as endocrine glands in the early stages of their development and later acquire exocrine functions as well In approximately 60 per cent of the cases studied allantochorionic pouches filled with endometrial cup secretion were found in juxtaposition with the cups The accumulated endometrial cup secretion indents the allantochorion (Fig 4) thus forming a sac which dangles into the allantochorionic cavity In those cases in which pouches are not formed the secretion and degenerated cups lie free in the space between the endometrium and the chorion The neck of the pouches tends to undergo con striction which if complete prevents absorption into the maternal circulation The secretion can then only be absorbed into the circu lation of the fetus where it may be responsible (Cole 1936 Rowlands 1947) for the transient but extraordinary interstitial hypertrophy of the gonads (Cole *et al* 1933) Amoroso and Rowlands (1951) hold that any PMS *which reaches the fetus is present in concentrations* too low to affect its gonadal development Such a view derives support from the observation of Catchpole and Lyons (1934) that extracts of whole fetus and of fetal liver only occasionally contain demonstrable PMS

A remarkable property of PMS which contributes to its prolonged effect is a relative inability to pass through the renal filter Although Zondek (1930) reported that the gonadotropin content of pregnant mares urine is sufficient for diagnostic purposes other investigators have failed to confirm his finding Quantitative data bearing on this point are meager However it has become clear that comparatively little of the hormone is excreted in the urine Blood levels of PMS were found to be high and urine levels low or nil in simultaneous samples obtained in 8 mares by Goss and Cole (1931) In one instance there were approximately 100 R U per cubic centimeter of serum and no detectable gonadotropic activity in 10 cc of urine Rats and rhesus monkeys injected with PMS excrete no gonad stimulating substances in their urine (Evans *et al* 1933)

Levels in Pregnancy

Qualitative tests for serum gonadotropic activity are of practical value in equine pregnancy diagnosis (Cole *et al* 1931 Hart and Cole 1932 Kust 1932 Wolters *et al* 1932 Glud *et al* 1933 Cromwell 1934 Kust 1934 Magnusson 1934 Catchpole 1935 Miller and Day 1939 Day and Miller 1940 Cole and Hart 1942 Mayer 1944 Roussel 1945 Zondek and Sulman 1945b 1947) Gonadotropin becomes detectable in the blood at approximately the fortieth day after the fertilizing coitus or at a fetal crown rump length of 2 0 cm Maximal concentrations of 70 000 to 450 000 I U per liter are reached between 55 and 80 days or at a fetal length of approximately 10 cm and are maintained for a comparatively short period The hormone disappears from the blood at approximately 150 days when the fetus has attained a crown rump length of 30 cm (Cole and Hart 1930a b Cole *et al* 1931 Catchpole and Lyons 1933 1934 Cole and Saunders 1935 Day and Rowlands 1940 Aylward and Ottoway 1945 Amoroso and Rowlands 1951 Clegg *et al* 1954)

Serum concentrations appear to be inversely proportional to the size of the mare (Cole 1938 Day and Rowlands 1940 1947 Rowlands 1949) The levels are extremely variable and bear no relation to the age or parity of the animal the diet or the concentration of gonadal hormones in the blood (Cole 1938 Day and Rowlands 1947 Rowlands 1949)

The gonadotropin concentration of the serum is uniformly high in twin pregnant mares having two sets of endometrial cups and generally exceeds that of mares carrying only one foal (Rowlands 1949) A sud

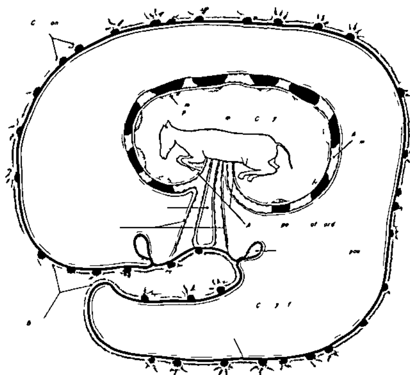


FIGURE 4 Diagram of the allantochorionic sac of the horse towards the middle of gestation (After Amoroso E C Placentation In Parkes A S (ed) *Physiology of Reproduction* II (3rd ed) London and New York Longmans Green and Co 1952)

Metabolism and Excretion

The limited data available concerning the metabolism of circulating PMS have been summarized by Zondek and Sulman (1945a) It was early noted (Cole *et al* 1932) that the biological response to a single injection of the hormone does not differ materially from the response to a number of divided doses i e within limits the response is independent of the degree of subdivision of the total dosage More direct evidence indicating that the destruction of PMS is comparatively slow has been obtained by studies of its disappearance rate in the rabbit and gelding (Catchpole *et al* 1935) The serum concentration of injected PMS was found to be reduced by approximately one half every 26 hours in the rabbit and every 6 days in the gelding There is no evidence that the hormone is excreted in rabbit urine or feces or that it is stored in the uterus spleen lungs kidneys or liver Since the disappearance rate is similar in spayed and intact rabbits *it is unlikely that the gonads play a significant role in its destruction*

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den fall in serum *potency occurs* after abortion (Aylward and Ottoway 1945 *Rowlands 1949*) The maintenance of a high blood level in an *instance* of fetal resorption suggests that the conceptus may be necessary for the preservation of the functional activity of the endometrial cups (Rowlands 1949)

The blood of the donkey contains demonstrable gonadotropic activity between the 40th and 200th day of pregnancy (Samodelkin 1939) However after the 150th to the 160th day of gestation the level falls too low for accurate pregnancy diagnosis (Svecin 1939)

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